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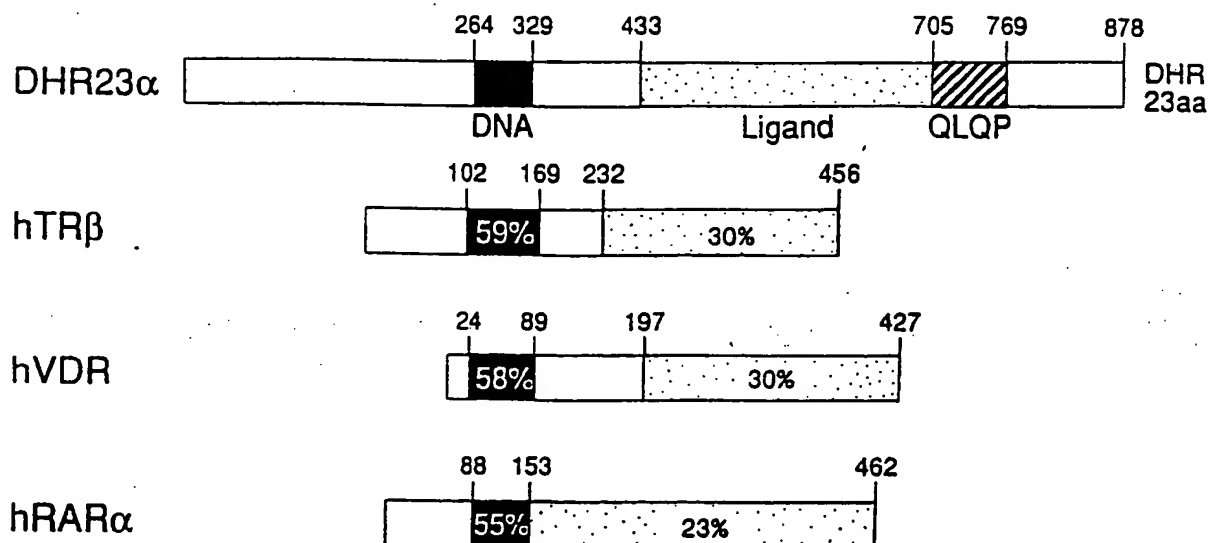
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(54) Title: ECDYSTEROID DEPENDENT REGULATION OF GENES IN MAMMALIAN CELLS



(57) Abstract

A method of inducing gene expression in a mammalian cell or an intact mammal comprising contacting an ecdysteroid, such as muristerone A, with an ecdysteroid receptor polypeptide within a mammalian cell, wherein said mammalian cell further contains a DNA binding sequence for said ecdysteroid receptor when in combination with its ligand, such as muristerone A, thereby resulting in gene expression.

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ECDYSTEROID DEPENDENT REGULATION OF GENES IN MAMMALIAN CELLS

5 FIELD OF THE INVENTION

This invention relates generally to the use of specific ecdysteroids in mammalian cells to induce gene expression of heterologous genes under the control of ecdysteroid receptor binding proteins.

BACKGROUND OF THE INVENTION

10 The steroid hormone receptor superfamily represents an evolutionarily conserved group of proteins that influence developmental and metabolic processes primarily by functioning as ligand-dependent transcription factors (for review, see K. R. Yamamoto, *Annu. Rev. Genet.* 19, 209 [1985]; M. Beato, *Cell* 56, 335 [1989]; R. M. Evans, *Science* 240, 889 [1988]). Structural analysis of receptor proteins has identified domains of these proteins that
15 function to bind DNA or ligand and to enhance transcription (S. Rusconi and K. R. Yamamoto, *EMBO J.* 6, 1309 [1987]; P. J. Godowski, D. Picard and K. R. Yamamoto, *Science* 241, 812 [1988]; S. M. Hollenberg and R. M. Evans, *Cell* 55, 899 [1988]; N. J. G. Webster, S. Green, J. R. Jin and P. Chambon, *Cell* 54, 199 [1988]). Ligand binding is required for the interaction of some
20 receptors with their cognate response elements (P. B. Becker, *et al.*, *Nature* 324, 686 [1988]) and may also control dimerization and transcriptional activation properties (S. Y. Tsai, *et al.*, *Cell* 55, 361 [1988]; V. Kumar and P. Chambon, *Cell* 55, 145 [1988]).

The ability of transcription factors to function in heterologous species has provided a system to analyze the functional domains of that factor and a novel mechanism to control the expression of heterologous genes (A. J. Courey and R. Tjian, *Cell* 55, 887 [1988]; A. J.
25 Courey, D. A. Holtzman, S. P. Jackson and R. Tjian, *Cell* 59, 827 [1989]). Mammalian steroid hormone receptors have been shown to function when expressed in yeast (D. Metzger, J. H. White and P. Chambon, *Nature* 334, 31 [1988]; D. Picard, M. Schena and K. R. Yamamoto, *Gene* 86, 257; M. Schena and K. R. Yamamoto, *Science* 241, 965 [1988]) and drosophila cells.

The ecdysteroids are steroids whose action is mediated by an intracellular receptor
30 and includes the molting hormones of insects. The ecdysteroid hormone 20-OH ecdysone, also known as β -ecdysone, controls timing of development in many insects. See, generally, Koolman (ed.), *Ecdysone: From Chemistry to Mode of Action*, Thieme Medical Pub., N.Y. (1989). The generic term "ecdysone" is frequently used as an abbreviation for 20-OH ecdysone. Pulses, or rises and falls, of the ecdysone concentration over a short period of time
35 in insect development are observed at various stages of Drosophila development.

These stages include embryogenesis, three larval stages and two pupal stages. The last pupal stage ends with the formation of the adult fly. One studied effect of ecdysone on development is that resulting from a pulse at the end of the third, or last, larval stage. This pulse triggers the beginning of the metamorphosis of the larva to the adult fly. Certain

tissues, called imaginal tissues, are induced to begin their formation of adult structures such as eyes, wings and legs.

During the larval stages of development, giant polytene chromosomes develop in the non-imaginal larval tissues. These cable-like chromosomes consist of aggregates comprising up to about 2,000 chromosomal copies. These chromosome aggregates are extremely useful because they provide the means whereby the position of a given gene within a chromosome can be determined to a very high degree of resolution, several orders of magnitude higher than is typically possible for normal chromosomes.

A "puff" in the polytene chromosomes is a localized expansion or swelling of these cable-like polytene chromosome aggregates that is associated with the transcription of a gene at the puff locus. A puff is, therefore, an indicator of the transcription of a gene located at a particular position in the chromosome.

A genetic regulatory model was proposed to explain the temporal sequence of polytene puffs induced by the ecdysone pulse which triggers the larval-to-adult metamorphosis. See, Ashburner *et al.*, "On the Temporal Control of Puffing Activity in Polytene Chromosomes," *Cold Spring Harbor Symp. Quant. Biol.* 38:655-662 (1974). This model proposed that ecdysone interacts reversibly with a receptor protein, the ecdysone receptor, to form an ecdysone-receptor complex. This complex would directly induce the transcription of a small set of "early" genes responsible for a half dozen immediately induced "early" puffs. These early genes are postulated to encode regulatory proteins that induce the transcription of a second set of "late" genes responsible for the formation of the "late" puffs that appear after the early puffs. The model thus defines a genetic regulatory hierarchy of three ranks, where the ecdysone receptor gene is in the first rank, the early genes in the second rank and the late genes in the third. While this model derived from the puffing pattern observed in a non-imaginal tissue, similar genetic regulatory hierarchies may also determine the metamorphic changes in development of the imaginal tissues that are also targets of ecdysone, as well as the changes in tissue development induced by the pulses of ecdysone that occur at other developmental stages.

Various structural data have been derived from vertebrate steroid and other lipophilic receptor proteins. A "superfamily" of such receptors has been defined on the basis of their structural similarities. See, Evans, "The Steroid and Thyroid Hormone Receptor Superfamily," *Science* 240:889-895 (1988); Green and Chambon, "Nuclear Receptors Enhance Our Understanding of Transcription Regulation," *Trends in Genetics* 4:309-314 (1988). Where their functions have been defined, these receptors, complexed with their respective hormones, regulate the transcription of their primary target genes, as proposed for the ecdysone receptor in the above model.

Ecdysteroid receptors from *Drosophila melanogaster* adult females were described by Handler *et al.* *Mol Cell Endo* 63:103-9, 1989. Ecdysteroid receptors for the blowfly *Calliphora vicina* were characterized by Lehmann *et al.*, *Eur J. Biochem* 181:577-82, 1989. The isolation of

20-hydroxyecdysone from *Vitex stricken* (East African herb) is described in JP1135794. The ecdysterone content of culture medium of *drosophila* salivary glands was described in SU1130605. Muristerone extracted from the seeds of *faladana* plants and used as a biological insecticide is described in U.S. Patent 3,828,082. The synthesis of ecdysone is described in U.S. Patent 3,354,154.

Analog of ecdysteroids may be produced by plants to disrupt the development of insects. One plant-produced ecdysteroid analog is meristone A. Muristerone or Muristerone A (Figure 7, Compound IV) has a molecular weight of 496.640 daltons, a formula of $C_{27}H_{44}O_8$ and a CHCD name of 2,3,5,11,14,20,22-Heptahydroxy-7-cholesten-6-one (Cononica, L *et al*, *Phytochemistry* 14: 525, 1975). It is a constituent of the plant *Ipomoea calonyction* and shows a high insect moulting activity. It is believed to protect the plant by disrupting insect development in the larval stage (Trematerra *et al*, *Bollettino Zool. Agr. Bachic* 18:87-93, [1986]). Muristerone A has been shown to have ecdysteroid activity in insects such as to trigger degeneration of the tick salivary gland (Lindsay *et al*, *J. Insect Physiol* 34:351-360, [1988]). Muristerone A-receptor complexes are not as sensitive to dissociation in high salt buffers as other ecdysteroid-receptor complexes and this affinity has allowed the use of radiolabeled muristerone A to follow ecdysteroid receptor during chromatography (Landon *et al*, *J. Biol Chem* 263:4693-4697, [1988]).

Many medically and commercially important proteins can be produced in a usable form by genetically engineered bacteria. However, many expressed proteins are processed incorrectly in bacteria and are preferably produced by genetically engineered eucaryotic cells. Typically, yeast cells or mammalian tissue-culture cells are used. Because it has been observed that protein processing of foreign proteins in yeast cells is also frequently inappropriate, mammalian cultured cells have become the central focus for protein production. It is common that the production of large amounts of foreign proteins makes these cells unhealthy, which may affect adversely the yield of the desired protein. This problem may be circumvented, in part, by using an inducible expression system. In such a system, the cells are engineered so that they do not express the foreign protein, and therefore are not unhealthy, until an inducing agent is added to the growth medium. In this way, large quantities of healthy cells can be produced and then induced to produce large amounts of the foreign protein. Unfortunately, in the presently available systems, the inducing agents themselves, such as metal ions or high temperature, adversely affect the cells, thus again lowering the yield of the desired foreign protein the cells produce. A need therefore exists for the development of innocuous inducing factors for efficient production of recombinant proteins. Such innocuous factors could also prove invaluable for human therapy, where the individual suffers from lack of the ability to produce particular proteins. By using methods similar to those for producing proteins in cultured cells, such innocuous factors for inducing the synthesis of the required protein could be used for controlling both the timing and the abundance of the protein produced

in the affected individual. Therefore, a need exists for an inducible expression system in both mammalian cell culture and in mammals *per se*.

The hormones that complex with mammalian or other vertebrate members of the steroid receptor superfamily are unlikely candidates for such innocuous factors, nor have they been found to satisfy the required properties of such factors, because mammalian cells contain these receptors, or highly homologous proteins, that would alter the expression of many target genes in the presence of the respective hormone, thereby adversely affecting the host cells. For these and other reasons, developing an alternative steroid receptor system has been a goal of researchers. Unfortunately, efforts have been unsuccessful despite significant investment of resources. The absence of information on the structure, function and molecular biology of non-mammalian steroid receptors has significantly hindered the ability to produce such products. Recently, the isolation and characterization of drosophila DHR23 α DNA, which contained a partial sequence of the DHR23 α polypeptide, a member of the steroid hormone receptor superfamily previously identified in the laboratory of Dr. David Hogness (W. Seegraves, thesis, Stanford University [1988]) allows the isolation of the DNA sequence encoding the DHR23 α . This isolated DNA sequence allows the construction of expression systems incorporating the DNA encoding the DHR23 α ecdysteroid receptor.

In summary, the insect steroid hormones and their receptors, such as the ecdysteroids, are a potential source of material for developing innocuous steroid receptor systems for use in mammalian cell culture and in mammals themselves. However, no ecdysteroid has been shown to function to induce activity of an ecdysteroid receptor in a mammalian cell. Therefore, to develop a system based upon an insect hormone-receptor system, there exists a need for an ecdysteroid hormone or hormone analog that induces the ecdysteroid receptor in mammalian cells to express those genes placed under control of the ecdysteroid receptor.

SUMMARY OF THE INVENTION

I show that DHR23 α , a Drosophila steroid receptor homologue, can function in cultured mammalian cells as an ecdysteroid-dependent transcription factor when induced by a specific group of ecdysteroids which includes muristerone A. Muristerone A and related ecdysteroids that lack a 25-hydroxyl group, will induce in mammalian cells the expression of DNA sequences under the transcriptional control of DHR23 α . DHR23 α inducible activity was not induced by any of the mammalian steroid hormones tested. The DNA-binding and transactivation activities of viral, mammalian or bacterial proteins were rendered ecdysteroid-dependent when fused to the DHR23 α ligand-binding domain. This system is useful in selectively regulating the expression of endogenous or heterologous genes in mammalian cells.

DESCRIPTION OF THE FIGURES

Figure. 1. (A) Nucleotide and derived amino acid sequence of the DHR23 α cDNA clone (seq. ID No. 1). Numbers on the left and right indicate nucleotide and amino acid residues, respectively. The conserved amino acids corresponding to the putative DNA-binding domain are underlined. (B) Schematic comparison of the *Drosophila* DHR23 α protein with the human thyroid hormone receptor (hTR β), the human vitamin D3 receptor (hVDR) and the human retinoic acid receptor (hRAR α). The amino acid residues are indicated by numbers above the boxes. The region of DHR23 α marked 'DNA' is compared with the DNA-binding domains of the other receptors. The region of DHR23 α marked 'Ligand' contains the highest region of homology to the ligand-binding domains of the other receptors. The percent identity in these regions is shown by the numbers within the boxes. The region labeled 'QLQP' is rich in the amino acids glutamine (Q) leucine (L) and proline (P).

Figure. 2. Specific ecdysteroids are agonists for the DHR23 α receptor in mammalian cells. Human 293 cells were cotransfected with 2.5 μ g of the expression plasmid pRSV.DHR23 α or the parental expression plasmid pRSV (Control) and 0.5 μ g of the reporter plasmid pEc₄M₇₇CO (EcRE) or pG₄M₇₇CO (GRE). As a control for transfection efficiency, 0.5 μ g of the control plasmid pRSV.hGH was included in the transfection mixture. After transfection, cells were treated without (-) or with alpha-ecdysone (alpha), 20-OH ecdysone (20-OH), polypodine B (ppB), ponasterone A (ponA) or muristeroine A (murA). CAT extracts were harvested 48 hrs after transfection and assayed. The values were normalized to the expression of hGH and the average values of three independent experiments are shown. The "fold induction" represents the level of expression of the reporter gene in cells incubated with hormone divided by the expression of that reporter gene in extracts from cells incubated in the absence of added ligand.

Figure. 3. Effect of mammalian hormones on activity of DHR23 α . Cells were transfected with the DHR23 α expression vector and Ec₄M₇₇CO reporter gene and treated without (-) or with the following hormones (1 μ M): muristerone A (mur A) dexamethasone (Dex) 17 β -estradiol (E2), aldosterone (Aldo), corticosterone (Cort) hydroxycorticosterone (OH-Cort) thyroid hormone (T3), promegestone (Promeg) or 1,25-dihydroxy vitamin D3 (VD3). Reporter gene activity was determined as described in Fig. 2.

Figure. 4. Schematic representation and expression of receptor proteins. (A) Receptor constructs are denoted according to a three part nomenclature describing the origin of their N-terminal transactivation, DNA-binding and ligand-binding domains. "G" and "Ec" refer to the glucocorticoid receptor and DHR23 α , respectively. "E" refers to a derivative of the rat glucocorticoid receptor DNA-binding domain with two amino acid substitutions (G458E, S459G) that convert the DNA-binding specificity to that of the estrogen receptor. "X" (solid box) indicates the DNA-binding domain (amino acids 1-87) of the *Escherichia coli* LexA protein. "V" (hatched box) denotes a derivative of the GR N-terminal domain in which amino acids 153-406 are replaced by the transcriptional activation domain of the HSV VP16 protein

(amino acids 411-490) GGEc was constructed by replacing the ligand-binding domain of GGG (amino acids 528-795) with the ligand-binding domain of DHR23 α (amino acids 329-878). Similarly, to construct GXEc, the ligand-binding domain of GXG was replaced with the DHR23 α ligand-binding domain. (B) Accumulation of receptor proteins in transfected cells.

- 5 Whole-cell extracts were prepared 48 hrs after transfection with receptor expression plasmids encoding the following fusion proteins. Lane 1, EcEcEc; lane 2, GGG; lane 3, GGEc; lane 4, VGEc; lane 5, G'E'G; lane 6, G'E'Ec; lane 7, GXG; lane 8, GXEc; lane 9, VXEc. The blots were reacted with monoclonal antibody B μ gR2 that recognizes an epitope in the N-terminal domain of the rat glucocorticoid receptor and then with a sheep antiserum to mouse antibody coupled to horseradish peroxidase. Positions of the molecular markers are indicated.

10 **Figure. 5.** RNase protection analysis of transcripts induced by receptor proteins. Total RNA was prepared from cells 48 hrs after transfection with expression plasmids encoding either DHR23 α (EcEcEc), GGG, or GGEc and the reporter gene G₄M-77GO. The position of 377 base protected band for G₄M-77CO and the 294 base protected band from the
15 internal control gene (expressed from a CMV enhancer/promoter construct) are indicated by the closed and opened arrows, respectively.

Figure. 6. Induction of estrogen receptors (ERs) by chimeric receptors. Cells were transfected with expression plasmids encoding either DHR23 α (EcEcEc), G'E'G or G'E'Ec and E₄M-77CO, that contains 4 ERs fused to the MTV promoter. Cells were
20 incubated for 48 hrs with or without (-) or with muristerone A (M) or dexamethasone (D).

Figure 7. The chemical structure of the ecdysteroids: (I) ecdysteroid numbering system, R₁ and R₂ are site of electronegative group substitution; (II) ecdysone; (III) 20-OH ecdysone(β -ecdysone); (IV) ponasterone A; (V) muristerone A; (VI) 5-dehydroxy muristerone A; (VII) and 11-dehydroxy muristerone A.

25

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Steroid receptors are members of a large family of transcription factors whose activity is tightly regulated by the binding of their cognate steroid ligand. These ligand-dependent transcription factors can be exploited to obtain the regulated expression of
30 heterologous genes in mammalian cells. However, the utility of these systems in transgenic animals is limited by the background of endogenous steroids and their receptors. The present invention demonstrates that an analog of the insect ecdysteroids, muristerone A, will induce gene expression in mammalian cells using the insect steroid receptor DHR23 α and its DNA binding sequence.

35 In the present invention, I wished to develop a system to specifically regulate heterologous genes in mammalian cells in tissue culture and in transgenic animals. Such a system provides a powerful method for regulating the synthesis of the products of heterologous genes. Systems that exploit the ability of mammalian steroid hormone receptors to function as ligand-dependent transcription factors have proved useful in regulating the

expression of heterologous genes in mammalian cells (D. I. Israel and R. J. Kaufman, *Nuc. Acids Res.* 17, 4589 [1989]). However, the applications of these systems in cultured cells or transgenic animals is limited by the background of endogenous steroid receptors and their ligands. In the present invention I show that the drosophila DHR23 α protein, a member of the steroid-hormone receptor superfamily previously identified in 1988 by W. Seegraves at Stanford University (W. Seegraves, thesis, Stanford University (1988)), can function as a ligand-dependent transcription factor in mammalian cells when induced by specific ecdysteroids such as DHR23 α . The activity of DHR23 α is induced upon administration of certain ecdysteroids but not by any of the mammalian hormones tested. Novel target gene specificity was obtained using chimeric receptors containing the DHR23 α ligand-binding domains fused to heterologous DNA-binding domains. Finally, the activity of these chimeric proteins could be increased by inclusion of a potent viral transactivation domain.

Oligonucleotide probes based on the partial DHR23 sequence (W. Seegraves, thesis, Stanford University (1988)) were used to screen a cDNA library prepared from drosophila early pupal larvae. The deduced amino acid sequence of the DHR23 α clone is shown in Fig. 1A. The highest region of homology between DHR23 α and other members of the steroid receptor superfamily is found in a cysteine-rich region (residues 264-329) that corresponds to the DNA-binding domain (Fig. 1B). The putative ligand-binding domain shares limited homology with members of the retinoic acid and thyroid hormone receptors, and the vitamin D receptor. The N-terminal domain (residues 1-263) does not share significant homology with any steroid receptor superfamily member. I also identified a second form of this protein, DHR23 β , that appears to arise by differential splicing. DHR23 β is identical to DHR23 α in the DNA and ligand-binding domains but contains an unrelated N-terminal domain of 234 amino acids.

DHR23 α has been reported to regulate transcription of genes containing ecdysone response elements (EcREs) in *drosophila* tissue culture cells treated with 20-OH ecdysone (M. Koelle, Paper presented at a seminar, 2 October 1989, Genentech, Inc., So. San Francisco, CA). I determined whether DHR23 α could function in mammalian cells treated with ecdysteroids to enhance the transcription of a reporter gene containing EcREs linked to the murine mammary tumor virus (MTV) promoter and chloramphenicol acetyltransferase (CAT) gene. Human 293 cells were cotransfected with an RSV-based expression vector that encodes DHR23 α and the reporter gene Ec₄M-77CO that contains 4 copies of an ecdysone response element (EcRE) from the *Drosophila* HSP27 promoter (G. Riddihough and H. R. B. Pelham, *EMBO J.* 6, 3729 (1987)) linked to an MTV promoter-CAT construct. CAT activity was determined in extracts from cells incubated with or without the ecdysteroids α - or 20-OH ecdysone, polypodine B, ponasterone A, or muristerone A. Neither α -ecdysone, 20-OH ecdysone, nor polypodine B acted as agonists for DHR23 α in mammalian cells. In contrast, expression of the reporter gene was markedly increased in cells treated with muristerone A and to a lesser extent with ponasterone A (Fig. 2). This induction was dependent on the presence of an EcRE in the reporter gene because DHR23 α did not regulate

expression of reporter genes containing binding sites for either the glucocorticoid receptor (GRE, Fig. 2) or for the estrogen receptor (ERE, Fig. 6). Thus, in mammalian cells DHR23 α acts in an ecdysteroid-dependent fashion to selectively stimulate expression from an EcRE containing reporter gene.

5 It is unclear why 20-OH-ecdysone and polypodine B, which are agonists in drosophila cell lines, fail to activate DHR23 α in mammalian cells. Transport failure, inactivation and non-specific binding may account for the lack of activity in mammalian cells. However, it has been shown that the specific activities and relative efficacies of ligands for their receptors sometimes differ when that receptor is expressed in a heterologous system. For example, it is
10 known that the activities of glucocorticoid ligands for their receptor differ significantly in yeast and mammalian cells. Surprisingly, only specific derivatives of ecdysone act as agonists, and these share the related feature of lacking the 25-hydroxyl group. The only structural difference between the weak agonist ponasterone A and 20-OH ecdysone, which is inactive, is that the former lacks a hydroxyl group at position 25. Even more surprising and
15 unexpected is that the strong agonist muristerone differs from ponasterone A solely by the addition of hydroxyl groups at positions 5 and 11. Therefore, surprisingly, in mammalian cells ecdysteroid inducers of DHR23 α -regulated expression lack a hydroxyl group at position 25. In a preferred embodiment, the ecdysteroid may contain one hydroxyl group at positions 5 or 11. Alternatively, any electronegative group substituted at positions 5 and/or 11 of
20 muristerone would be expected to result in activation of ecdysteroid receptors. Among the electronegative groups that may be substituted at positions 5 and 11 of muristerone are hydroxyl, ketone, sulfhydryl, nitrate, nitrite, and halogens, particularly fluorine, bromine and chlorine. The most active inducer of DHR23 α regulated expression is muristerone A, which contains hydroxyl groups at both the 5 and 11 positions. The CHCD name for muristerone A
25 is 2,3,5,11,14,20,22-heptahydroxy-7-cholesten-6-one; for 5-dehydroxy muristerone A it is 2,3,11,14,20,22-septahydroxy-7-cholesten-6-one; and for 11-dehydroxy muristerone A it is 2,3,5,14,20,22-septahydroxy-7-cholesten-6-one.

 In the present invention, ecdysteroid receptor is defined as any insect steroid receptor that has a physiologically significant binding affinity for muristerone A, or a derivative of
30 muristerone A which contains an electronegative substitution at the 5 or 11 positions. Among such physiologically significant receptors are the ecdysone receptor and DHR23X. A physiologically significant binding affinity is effective binding at a concentration of 10^{-6} molar or less. In the present invention, ecdysteroid is defined as any steroid lacking 25-OH having a physiologically effective affinity for an ecdysteroid receptor.

35 In order to determine if mammalian steroid hormones could act as agonists for DHR23 α , I tested representative members of hormones known to activate mammalian steroid receptors. None of these hormones could act as agonists for DHR23 α (Fig. 3). These results suggest that DHR23 activity is selectively regulated in mammalian cells by ecdysteroids.

Therefore, mammalian cells containing genes under the control of DHR23 α will be induced by muristerone A, and not by those mammalian steroid hormones tested.

Ecdysteroid regulation of mammalian, viral and bacterial DNA binding or transactivation domains.

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A remarkable feature of steroid hormone receptors is the degree to which individual domains can function when combined with domains of heterologous proteins. The DNA binding specificity of a receptor can be altered by replacing its DNA-binding domain with those of other steroid receptors (S. Green and P. Chambon, *Proc. Natl. Acad. Sci. U.S.A.* **325**, 75 [1987]) or from bacterial (P. J. Godowski, D. Picard and K. R. Yamamoto, *Science* **241**, 812 [1988]) or yeast (N. J. G. Green, S. Green, J. R. Kin and P. Chambon, *Cell* **54**, 199 [1988]) DNA-binding proteins. In some cases, the activities of heterologous proteins become hormone regulated if that protein is fused to a steroid receptor ligand-binding domain. For example, the transactivation or transformation activities of *E1A*, *c-myc* or *c-fos* can be brought under hormonal control by fusion of a steroid receptor ligand-binding domain (M. Eilers, D. Picard, K. R. Yamamoto and J. M. Bishop, *Nature* **340**, 66 [1989]; D. Picard, S.J. Salser and K. R. Yamamoto, *Cell* **54**, 1073 [1988]; G. Superti-Furga, G. Bergers, D. Picard and M. Busslinger, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5114 [1991]).

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I choose to determine if the ligand-binding domain of DHR23 α could be used to regulate the DNA-binding and transactivation domains of the mammalian glucocorticoid receptor for the following reasons. First, in contrast to DHR23 α , high affinity DNA-binding sites for the GR have been identified (M. Beato, *Cell* **56**, 335 [1989]) and reporter genes containing these sites are very strongly regulated. Thus, the sensitivity of our assays could be increased. Secondly, these chimeric receptors could be utilized to selectively regulate endogenous genes in mammalian cells in response to ecdysteroids. I constructed a chimeric gene, GGEC, in which the sequences encoding the ligand-binding domain of the GR were replaced with that of DHR23 α (Fig. 4A). Western blot analysis indicated that GGEC was expressed in transfected cells, although at considerably lower levels than the intact GR (Fig. 4B). As expected, DHR23 α failed to induce the expression of G₄M-77CO, a reporter gene that contains 4 copies of a high affinity GRE (Table 1). However, expression of the GRE-containing reporter gene was induced more than 700 fold by GGEC in muristerone-treated cells. The relative efficacies of ecdysteroids as agonists for the chimeric receptor are identical to that as for DHR23 α ; neither α -ecdysone, 20-OH ecdysone or polypodine B acted as agonists whereas ponasterone A and muristerone A acted as weak and strong agonists respectively.

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Results of the CAT assays were confirmed and extended by direct analysis of transcripts initiated at the regulated promoter. RNA isolated from transfected cells was assayed by RNase mapping experiments using probes complementary to the GRE containing reporter plasmid and a cotransfected control gene expressed from the CMV enhancer and

promoter. Figure 5 shows that GGEC acts in a hormone-dependent fashion to stimulate expression from a GRE-MTV promoter construct.

I then determined if a DHR23 fusion protein could regulate genes normally responsive to estrogens. I constructed a derivative of GGEC that incorporates a two amino acid change in the first finger of the rat glucocorticoid receptor (G458E, S459G) that has been shown to convert the DNA-binding specificity of the GR to that of the ER (K. Umesono and R. Evans, *Cell* 57, 1139 [1989]; M. Danielsen, L. Hinck, G. Ringold, *Cell* 57, 1131 [1989]) (Fig. 4A). This fusion protein, G*E*Ec, now regulates the ERE containing reporter gene E4M-77CO in a muristerone-dependent fashion (Figure 6). As expected, G*E*Ec failed to induce the expression of reporter genes lacking EREs

Chimeric proteins containing the DHR23 α ligand-binding domain fused to the DNA-binding domains of mammalian proteins are expected to prove useful in regulating the expression of either native or transgenic endogenous genes in transgenic animals. Such fusion constructs are expected to be useful in regulating the expression of exogenous genes introduced into transgenic mammals or mammalian cells. I next determined if the DHR23 α ligand-binding domain could be used to regulate the activity of a DNA-binding domain not normally expressed in mammalian cells. I constructed the chimeric gene GLxEc by replacing the sequences coding for the GR DNA-binding domain in the GGEC fusion with those encoding the DNA-binding domain of the *Escherichia coli* LexA repressor (J. W. Little and S. A. Hill, *Proc. Natl. Acad. Sci. U.S.A.* 82, 2301 [1985]) (Fig. 4). A reporter gene, X₄C-33CO was constructed that contains 4 copies of a 26-bp lex operator (R. Brent and M. Ptashne, *Nature* 312, 612 [1984]) at position -33 of the CMV promoter. GLxEc had no effect on the expression of a OC-33CO, a reporter gene lacking the lex operator (Fig. 6B). However, transcription of X₄C-33CO was strongly induced by GLxEc, and this induction was fully hormone-dependent (Fig. 6A). As controls I showed that X₄C-33CO was not induced in cells treated with muristerone and cotransfected with either DHR23 α , that lacks the lexA DNA-binding domain, or by GLxG (P. J. Godowski, D. Picard and K. R. Yamamoto, *Science* 241, 812 [1988]) that contains the glucocorticoid receptor ligand-binding domain.

Finally, I determined if the activity of DHR23 α fusion proteins could be further enhanced by inclusion of a potent viral transactivation domain. I constructed VGEc and VLxEc fusion genes by replacing a portion of the GR N-terminal activation domain in GGEC and GLxEc, respectively, with the herpes virus VP16 acidic activation domain (I. Sadowski *et al.*, *Proc. Natl. Acad. Sci.* 335, 563 [1988]; D. J. Cousens, R. Greaves, C. R. Coding, and P. O'Hare, *EMBO J.* 8, 2337 [1989]) (Fig. 4A). In transfected cells, these proteins accumulated to similar levels as derivatives lacking the VP16 activation domain (Fig. 4B). Both VGEc and VLxEc acted in an ecdysteroid-dependent fashion to induce activity of the appropriate reporter gene (Tables 1 and 2). However, the activity of VGEc and VLxEc was 5 and 10 fold greater than GGEC and GLxEc, respectively (M. A. Labow, S.B. Baim, T. Shenk, and A. J. Levine, *Mol. and Cell. Biol.* 10, 3343 [1990]). Thus, the DHR23 α ligand-binding domain can be

used to regulate the activities of viral, mammalian and bacterial DNA-binding or transactivation domains.

The development of a system for regulated expression of endogenous and exogenous genes in eukaryotic cells provides an important method to study the function of those gene products and to develop animals models for disease. Our results demonstrate the feasibility of using nonmammalian steroid hormone receptors to regulate genes in mammalian cells. There are several important features of this system. DHR23 α acts as a potent and selective regulator of the transcription of genes containing EcREs', and the activity of DHR23 α can be further modified by replacing its DNA-binding or transactivation domains with those from heterologous proteins. Importantly, DHR23 α activity is regulated by ecdysteroids, which are not normally expressed in mammalian cells. Although I did not survey all of the mammalian steroids, none of those representing the most abundant of the natural murine steroids acted as agonists for DHR23 α . Thus, it is conceivable that the transcriptional regulatory activities of DHR23 α or DHR23 α fusion proteins will be completely dependent on administration of exogenous ligand.

Several reports have demonstrated the feasibility of using the *E. coli* lac repressor to regulate gene expression in mammalian cells. Both the lac repressor and the steroid receptor based systems can induce or repress (D. M. Omitz, R. W. Moreadith and Leder P., *PNAS* 88, 698 [1991]) transcription. One attractive feature of a steroid receptor based regulatory system is the remarkable flexibility in the types of activities that can be controlled by the ligand-binding domain. The activities of structurally distinct DNA-binding proteins such as *lexA*, *c-fos*, GAL4 and *c-myc* are rendered hormone-dependent when fused to a steroid receptor ligand-binding domain (P. J. Godowski, D. Picard and K. R. Yamamoto, *Science* 241, 812 [1988]; N. J. G. Green, S. Green, J. R. Kin and P. Chambon, *Cell* 54, 199 [1988]; M. Eilers, D. Picard, K. R. Yamamoto and J. M. Bishop, *Nature* 340, 66 [1989]; D. Picard, S.J. Salser and K. R. Yamamoto, *Cell* 54, 1073 [1988]; G. Superti-Furga, G. Bergers, D. Picard and M. Busslinger, *Proc. Natl. Acad. Sci. U.S.A.* 88, 5114 [1991]). Chimeric proteins can be further modified by addition or subtraction of transactivation domains. Thus, it is expected that DHR23 α fusion genes can be constructed to regulate the expression of virtually any gene for which a cis-acting regulatory sequence and its cognate DNA-binding domain have been identified.

Among the genes that are anticipated for use in the method of the present invention are those encoding cytokines, hormones, structural proteins, enzymes and nucleic acids that are present in less than therapeutically needed concentrations in mammals. Among the cytokines and hormones are polypeptides such as: growth hormone, insulin-like growth factors, interleukins, human growth hormone, N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), parathyroid hormone, and leutinizing hormone (LH), hemopoietic growth factor, hepatic growth

factor, fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor-alpha and -beta, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, glucagon, inhibin, activin, vascular endothelial growth factor, integrin, thrombopoietin, erythropoietin, nerve growth factors such as NGF- β , platelet-growth factor, hemopoietic growth factor, tissue factor protein, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, transforming growth factors (TGF) such as TGF-alpha and TGF-beta, insulin-like growth factor-I and -II, latency associated peptide, erythropoietin, osteoinductive factors, osteoinductive factors, interferons such as interferon-alpha, -beta, and -gamma, colony stimulating factors (CSFs) such as M-CSF, GM-CSF, and G-CSF, interleukins (ILs) such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 and other polypeptide factors.

Among the enzymes are tissue plasminogen activase (TPA), urokinase, elastase, adenosine deaminase, bombesin, factor IX, factor VIII, thrombin, enkephalinase, β -lactamase, superoxide dismutase, reverse transcriptase, RNase, DNase, enzymes involved in glycolysis, Krebs's cycle, gluconeogenesis, urea cycle, oxidative phosphorylation, the synthesis or degradation of purines, pyrimidines, nucleic acid polymers, cholesterol, protein, glycogen, fatty acids, carbohydrate, phospholipids, glycoproteins or lipids.

Among the structural proteins are lung surfactant, albumin, antibodies, carrier proteins, glycoprotein hormone receptors, CD-4, insulin-like growth factor binding proteins, calcitonin, factor VIII, an antibody, protein A or D, rheumatoid factors, viral antigens, HIV envelope proteins GP120 and GP140, immunoglobulins, serum proteins and any protein present in a suboptimal amount.

Among the nucleic acids are DNA and RNA, both sense and antisense strands. The RNA may be any type, including ribosomal RNA, messenger RNA, transfer RNA, small nuclear RNA and RNA which has an enzymatic function or participates in an enzymatic function. The methods of the present invention have application to the production of nucleic acids which act as antisense or suppressor nucleic acids and inhibit the activity of a naturally occurring mammalian nucleic acid. Such nucleic acids have particular therapeutic efficacy in treating viral diseases, cancers and excessive production of a protein or other metabolic product.

Omiz, *et al.* (D. M. Omiz, R. W. Moreadith and Leder P., *PNAS* 88, 698 ([1991]) have described a binary system for regulating expression of heterologous genes in transgenic mice. In this system, a "transactivator" strain expressing the yeast GAL4 protein is crossed with "target" strains containing a transcriptionally silent transgene controlled by UAS sequences. The bigenic progeny of this cross express both transgenes in the same tissue. Bigenic systems incorporating DHR23 α or DHR23 α gene-fusions will provide a general method to control the abundance, time course and/or tissue specific expression of endogenous or exogenous genes. For example, tissue specific and developmentally regulated expression of transgenes controlled by EcREs is expected to be achieved by targeting the expression DHR23 α with appropriate tissue specific enhancers. The expression of the EcRE containing

"target" gene is expected to be induced at appropriate times by "turning-on" its activator with ecdysteroids. This development of a system that allows tissue and developmental control of genes in transgenic animals provides an important approach that complements strategies based on "gene-knockout" technology.

5 Mammalian cells suitable for the present method include those contained in an intact mammal. They include both differentiated and undifferentiated cells. Also included within the term mammalian cells are established cell lines and primary cell cultures derived from mammalian tissue. Among the preferred mammalian cell lines are 293 cell line and CHO cell line.

10

Therapeutic Compositions and Administration of Ecdysteroid

Ecdysteroids may be administered to transgenic mammals or mammalian cells that contain a gene under the transcriptional control of an ecdysteroid. For example, gene therapy to enable a mammal to express a polypeptide previously produced in insufficient quantities.

15 Examples of such polypeptides are described above.

Therapeutic formulations of ecdysteroids are prepared for storage by mixing ecdysteroid subunit having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*Remington's Pharmaceutical Sciences, supra*), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are
20 nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and
25 other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

The ecdysteroid to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following
30 lyophilization and reconstitution. The ecdysteroid ordinarily will be stored in lyophilized form or in solution. Therapeutic ecdysteroid compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of ecdysteroid administration is in accord with known methods, e.g. injection
35 or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems as noted below. The ecdysteroid is administered continuously by infusion or by bolus injection.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the ecdysteroid, which matrices are in the form of

shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed. Mater. Res.* 15: 167-277 (1981) and Langer, *Chem. Tech.* 12: 98-105 (1982) or poly(vinylalcohol), polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman *et al.*, *Biopolymers* 22: 547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron DepotTM (injectable micropheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

Sustained-release ecdysteroid compositions also include liposomally entrapped ecdysteroid. Liposomes containing ecdysteroid are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, *Proc. Natl. Acad. Sci.* 82: 3688-3692 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci.* 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal ecdysteroid therapy.

An effective amount of ecdysteroid to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, the activity of the polypeptide induced by the ecdysteroid and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer the ecdysteroid until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays for the presence or activity of the induced polypeptide.

Ecdysteroid compositions are administered to transgenic mammals, or to transgenic mammalian cell culture, when there is within the target cell population an available ecdysteroid receptor and an ecdysteroid DNA-binding domain positioned to promote the expression of a DNA sequence encoding a desired polypeptide or nucleic acid. The ecdysteroid receptor, such as DHR23α, acts as a potent and selective regulator of the transcription of genes containing ecdysteroid receptor DNA-binding sites. The activity of ecdysteroids, such as DHR23α, can be further modified by replacing its DNA-binding or transactivation domains with those from other naturally occurring genes, thereby facilitating ecdysteroid control over the other naturally occurring genes and the products they produce. The chimeric ecdysteroid receptors still bind the same ligand, however, they induce expression of the gene specified by the heterologous DNA binding domain specified by fused transactivation domain. Transformation of mammalian cells is accomplished using standard methods. Vectors suitable for transforming mammalian cells include viral and mammalian DNA capable of expression in mammalian cells.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

CLONING OF DNA ENCODING DHR23 α

Oligonucleotide probes based on the partial DHR23 sequence (W. Seegraves, thesis, Stanford University [1988]) were used to screen a cDNA library prepared from *Drosophila* early pupal larvae. The deduced amino acid sequence of the DHR23 α clone is shown in Fig. 1A. The highest region of homology between DHR23 α and other members of the steroid receptor superfamily is found in a cysteine-rich region (residues 264-329) that corresponds to the DNA-binding domain (Fig. 1B). The putative ligand-binding domain shares limited homology with members of the retinoic acid and thyroid hormone receptors, and the vitamin D receptor. The N-terminal domain (residues 1-263) does not share significant homology with any steroid receptor superfamily member. I also identified a second form of this protein, DHR23 β , that appears to arise by differential splicing. DHR23 β is identical to DHR23 α in the DNA and ligand-binding domains but contains an unrelated N-terminal domain of 234 amino acids.

Figure 1 A illustrates the nucleotide and derived amino acid sequence of the DHR23 α cDNA clone. Numbers on the left and right indicate nucleotide and amino acid residues, respectively. The first ATG following an upstream, in-frame stop codon is underlined and was chosen as the initiating codon. The initiator methionine conforms to the *Drosophila* consensus sequence for translation initiation. The conserved amino acids corresponding to the putative DNA-binding domain are underlined. To isolate the full-length DHR23 α clones, ~600,000 phage from a *Drosophila* third instar larval library were screened with two 50-mer oligonucleotide probes corresponding to nucleotides 109-158 and 1820-1869 of the partial DHR23 α sequence in Seegraves, Stanford University Thesis, 1988. Eight positive clones were isolated, further characterized by PCR analysis and the nucleotide sequence of the two largest inserts was determined. The results of the sequencing are shown in figure 1A.

EXAMPLE 2

EXPRESSION AND ACTIVITY OF DHR23 α POLYPEPTIDE IN MAMMALIAN CELLS

I determined whether DHR23 α could function in mammalian cells treated with ecdysteroids to enhance the transcription of a reporter gene containing EcREs linked to the murine mammary tumor virus (MTV) promoter and chloramphenicol acetyltransferase (CAT) gene. Human 293 cells were cotransfected with an RSV-based expression vector that encodes DHR23 α and the reporter gene Ec4M.77CO that contains 4 copies of an ecdysone response element (EcRE) from the *Drosophila* HSP27 promoter (G. Riddihough and H. R. B. Pelham, *EMBO J.* 6, 3729 [1987]) linked to an MTV promoter-CAT construct. CAT activity was determined in extracts from cells incubated with or without the ecdysteroids α - or 20-OH ecdysone, polypodine B, ponasterone A, or muristerone A. Neither α -ecdysone, 20-

OH ecdysone, nor polypodine B acted as agonists for DHR23 α in mammalian cells. In contrast, expression of the reporter gene was markedly increased in cells treated with muristerone A and to a lesser extent with ponasterone A (Fig. 2). This induction was dependent on the presence of an EcRE in the reporter gene because DHR23 α did not regulate expression of reporter genes containing binding-sites-for-either-the-glucocorticoid-receptor (GRE, Fig. 2) or for the estrogen receptor (ERE, Fig. 6). Thus, in mammalian cells DHR23 α acts in an ecdysteroid-dependent fashion to selectively stimulate expression from an EcRE containing reporter gene.

Specific ecdysteroids are agonists for the DHR23 α receptor in mammalian cells. Human 293 cells were cotransfected with 2.5 μ g of the expression plasmid pRSV.DHR23 α or the parental expression plasmid pRSV (Control) and 0.5 μ g of the reporter plasmid pEc₄M.₇₇CO (EcRE) or pG₄M.₇₇CO (GRE). As a control for transfection efficiency, 0.5 μ g of the control plasmid pRSV.hGH was included in the transfection mixture. After transfection, cells were treated without (-) or with alpha-ecdysone (alpha), 20-OH ecdysone (20-OH), polypodine B (ppB), ponasterone A (ponA) or muristerone A (murA). All candidate ligands were added at a concentration of 1 μ M. CAT extracts were harvested 48 hrs after transfection, and assays were performed as described (D. R. Cavener, *Nucl. Acids Res.* 15, 1353-1361 [1987]). The values were normalized to the expression of hGH and the average values of three independent experiments are shown in Figure 2. The "fold induction" represents the level of expression of the reporter gene in cells incubated with hormone divided by the expression of that reporter gene in extracts from cells incubated in the absence of added ligand.

The effect of mammalian hormones on activity of DHR23 α is shown in Figure 3. Cells were transfected with the DHR23 α expression vector and Ec₄M.₇₇CO reporter gene and treated without (-) or with the following hormones (1 μ M): muristerone A (mur A) dexamethasone (Dex) 17 β -estradiol (E2), aldosterone (Aldo), corticosterone (Cort) hydroxycorticosterone (OH-Cort) thyroid hormone (T3), promegestone (Promeg) or 1,25-dihydroxy vitamin D3 (VD3). Reporter gene activity was determined as described above for Figure 2. Retinoic Acid also did not act as an agonist for DHR23 α .

A schematic representation of chimeric genetic constructs and a Western blot of the resulting expressed receptor proteins are illustrated in figures 4A and 1B. In Figure 4A receptor constructs are denoted according to a three-part nomenclature describing the origin of their N-terminal transactivation, DNA-binding and ligand-binding domains. "G" and "Ec" refer to the glucocorticoid receptor and DHR23 α , respectively. "E" refers to a derivative of the rat glucocorticoid receptor DNA-binding domain with two amino acid substitutions (G458E, S459G) that convert the DNA-binding specificity to that of the estrogen receptor (K. Umesono and R. Evans, *Cell* 57, 1139 [1989]; M. Danielsen, L. Hinck, G. Ringold, *Cell* 57, 1131 [1989]). "X" (solid box) indicates the DNA-binding domain (amino acids 1-87) of the *Escherichia coli* LexA protein. The "V" (hatched box) denotes a derivative of the GR N-terminal domain in which amino acids 153-406 are replaced by the transcriptional activation domain of the

HSV VP16 protein (amino acids 411-490) The construct GGEc was constructed by replacing the ligand-binding domain of GGG (amino acids 528-795) with the ligand-binding domain of DHR23 α (amino acids 329-878). Similarly, to construct GXEc, the ligand-binding domain of GXG (referred to as NLxC in P. J. Godowski, D. Picard and K. R. Yamamoto, *Science* **241**, 812 [1988]) was replaced with the DHR23 α ligand-binding domain.

In figure 4B, the accumulation of receptor proteins in transfected cells are shown in a Western blot detected with enzyme-linked antibodies. Whole cell extracts were prepared 48 hrs after transfection with receptor expression plasmids encoding the following fusion proteins. Lane 1, EcEcEc; lane 2, GGG; lane 3, GGEc; lane 4, VGEc; lane 5, G"E"G; lane 6, G"E"Ec; lane 7, GXG; lane 8, GXEc; lane 9, VXEc. The blots were reacted with monoclonal antibody BuGR2, that recognizes an epitope in the N-terminal domain of the rat glucocorticoid receptor (30) and then with a sheep antiserum to mouse antibody coupled to horseradish peroxidase. Positions of the molecular markers are indicated.

RNase protection analysis of transcripts induced by receptor proteins is shown in Figure 5. Total RNA was prepared from cells 48 hrs after transfection with expression plasmids encoding either DHR23 α (EcEcEc), GGG, or GGEc and the reporter gene G₄M-77GO. This reporter gene contains 4 GREs fused at position -77 of an MTV promoter human growth hormone gene construct. Assays used 50 μ gs of total RNA. The position of 377 base protected band for G₄M-77CO and the 294 base protected band from the internal control gene (expressed from a CMV enhancer/promoter construct) are indicated by the closed and opened arrows, respectively.

The induction of EREs by chimeric receptors is shown in Figure 6. Cells were transfected with expression plasmids encoding either DHR23 α (EcEcEc), G"E"G or G"E"Ec and E₄M-77CO, that contains 4 EREs fused to the MTV promoter. Cells were incubated for 48 hrs with or without (-) or with muristerone A (M) or dexamethasone (D). Reporter gene activity was determined as described above for Figure 2.

Illustrated in Table 1 below is the induction of a glucocorticoid receptor (GRE) responsive gene by chimeric receptors. 293 cells were transfected with effector plasmids encoding the indicated receptor proteins and the reporter gene G₄M-77CO. These transfected cells were incubated either without added hormone (-), with muristerone A (M) or with dex (D). The reporter gene activity was determined as described above for Fig. 2. The "fold induction" represents the level of expression of the reporter gene in cells incubated with hormone divided by the expression of that reporter gene in extracts from cells incubated in the absence of added ligand.

Table 1
Induction of Glucocorticoid Receptor By Chimeric receptors

5	Receptor			
		-	M	D
	EcEcEc	1.0 (0.2)	0.9 (0.2)	NT
	GGG	1.0 (0.3)	1.0 (0.3)	347
	GGEc	1.7 (0.3)	798 (57)	NT
10	VGEc	4.7 (0.8)	3117 (240)	NT

Illustrated in Table 2 is the transcriptional activation by receptor-LexA fusion proteins. Effector plasmids encoding the indicated receptor proteins were transfected with a reporter gene either containing (X₄C-₃₃CO) or lacking (OC-₃₃CO) lex-operators. "Control" indicates the cells were transfected with an "effector" plasmid which does not contain a receptor cDNA. The cells were incubated either with (+) or without (-) muristerone A. The fold induction was determined as described previously; the standard deviations are shown in parenthesis.

Table 2
Transcriptional Activation By Chimeric Receptors

25	Receptor	X ₄ C- ₃₃ CO		OC- ₃₃ CO	
		-	+	-	+
	Control	1.0 (0.3)	1.1 (0.3)	1.0 (0.2)	1.1 (0.4)
	EcEcEc	1.2 (0.2)	1.4 (0.1)	NT	NT
	GXG	1.0 (0.1)	1.1 (0.2)	NT	NT
	GXEc	1.0 (0.2)	44.3 (8.3)	0.7 (0.2)	0.9 (0.3)
30	VXEc	4.7 (1.3)	563 (61)	0.8 (0.4)	1.0 (0.2)

While the invention has necessarily been described in conjunction with preferred embodiments, one of ordinary skill, after reading the foregoing specification, will be able to

effect various changes, substitutions of equivalents, and alterations to the subject matter set forth herein, without departing from the spirit and scope thereof. Hence, the invention can be practiced in ways other than those specifically described herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the appended claims and
5 equivalents thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT: Genentech, Inc.

 (ii) TITLE OF INVENTION: Ecdyst roid Dependent Regulation of Genes
In Mammalian Cells

10 (iii) NUMBER OF SEQUENCES: 1

 (iv) CORRESPONDENCE ADDRESS:

15 (A) ADDRESSEE: Genentech, Inc.
 (B) STREET: 460 Point San Bruno Blvd
 (C) CITY: South San Francisco
 (D) STATE: California
 (E) COUNTRY: USA
 (F) ZIP: 94080

20 (v) COMPUTER READABLE FORM:

 (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: patin (Genentech)

25

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 (A) APPLICATION NUMBER:
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30

 (viii) ATTORNEY/AGENT INFORMATION:

 (A) NAME: Fitts, Renee A.
 (B) REGISTRATION NUMBER: 35,136
 (C) REFERENCE/DOCKET NUMBER: 607

35

 (ix) TELECOMMUNICATION INFORMATION:

 (A) TELEPHONE: 415/225-1489
 (B) TELEFAX: 415/952-9881
 (C) TELEX: 910/371-7168

40

(2) INFORMATION FOR SEQ ID NO:1:

 (i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 2970 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5
AAGCTTCTTG TCCCCAGCCG ACGCTAAGTG AACGGAAAAC GGCCACAAAA 50

10
CGGCGACTAT CGGCTGCCAG AGG ATG AAG CGG CGC TGG TCG 91
Met Lys Arg Arg Trp Ser
1 5

15
AAC AAC GGC GGC TTC ATG CGC CTA CCG GAG GAG TCG TCC 130
Asn Asn Gly Gly Phe Met Arg Leu Pro Glu Glu Ser Ser
10 15

20
TCG GAG GTC ACG TCC TCC TCG AAC GGG CTC GTC CTG CCC 169
Ser Glu Val Thr Ser Ser Ser Asn Gly Leu Val Leu Pro
20 25 30

25
TCG GGG GTG AAC ATG TCG CCC TCG TCG CTG GAC TCG CAC 208
Ser Gly Val Asn Met Ser Pro Ser Ser Leu Asp Ser His
35 40 45

30
GAC TAT TGC GAT CAG GAC CTT TGG CTC TGC GGC AAC GAG 247
Asp Tyr Cys Asp Gln Asp Leu Trp Leu Cys Gly Asn Glu
50 55

35
TCC GGT TCG TTT GGC GGC TCC AAC GGC CAT GGC CTA AAT 286
Ser Gly Ser Phe Gly Gly Ser Asn Gly His Gly Leu Asn
60 65 70

40
CAG CAG CAG CAG AGC GTC ATC ACG CTG GCC ATG CAC GGG 325
Gln Gln Gln Gln Ser Val Ile Thr Leu Ala Met His Gly
75 80

45
TGC TCC AGC ACT CTG CCC GCG CAG ACA ACC ATC ATT CCG 364
Cys Ser Ser Thr Leu Pro Ala Gln Thr Thr Ile Ile Pro
85 90 95

50
ATC AAC GGC AAC GCG AAT GGG AAT GGA GGC TCC ACC AAT 403
Ile Asn Gly Asn Ala Asn Gly Asn Gly Gly Ser Thr Asn
100 105 110

55
GGC CAA TAT GTG CCG GGT GCC ACC AAT CTG GGA GCG TTG 442
Gly Gln Tyr Val Pro Gly Ala Thr Asn Leu Gly Ala Leu
115 120

60
GCC AAC GGG ATG CTC AAT GGG GGC CTC AAT GGA ATG CAG 481
Ala Asn Gly Met Leu Asn Gly Gly Leu Asn Gly Met Gln
125 130 135

CAA CAG ATT CAG AAT GGC CAC GGC CTC ATC AAC TCC ACA 520
Gln Gln Ile Gln Asn Gly His Gly Leu Ile Asn Ser Thr
140 145

ACG CCC TCA ACG CCG ACC ACC CCG CTC CAC CTT CAG CAG 559
Thr Pro Ser Thr Pro Thr Thr Pro Leu His Leu Gln Gln
150 155 160

AAC CTG GGG GGC GCG GGC GGC GGC GGT ATC GGG GGA ATG 598
Asn Leu Gly Gly Ala Gly Gly Gly Gly Il Gly Gly Met
165 170 175

	GGT ATT CTT CAC CAC GCG AAT GGC ACC CCA AAT GGC CTT 537 Gly Ile Leu His His Ala Asn Gly Thr Pro Asn Gly Leu 180 185
5	ATC GGA GTT GTG GGA GGC GGC GGC GGA GTA GGT CTT GGA 676 Ile Gly Val Val Gly Gly Gly Gly Gly Val Gly Leu Gly 190 195 200
10	GTA GGC GGA GGC GGA GTG GGA GGC CTG GGA ATG CAG CAC 715 Val Gly Gly Gly Gly Val Gly Gly Leu Gly Met Gln His 205 210
15	ACA CCC CGA AGC GAT TCG GTG AAT TCT ATA TCT TCA GGT 754 Thr Pro Arg Ser Asp Ser Val Asn Ser Ile Ser Ser Gly 215 220 225
20	CGC GAT GAT CTC TCG CCT TCG AGC AGC TTG AAC GGA TAC 793 Arg Asp Asp Leu Ser Pro Ser Ser Ser Leu Asn Gly Tyr 230 235 240
25	TCG GCG AAC GAA AGC TGC GAT GCG AAG AAG AGC AAG AAG 832 Ser Ala Asn Glu Ser Cys Asp Ala Lys Lys Ser Lys Lys 245 250
30	GGA CCT GCG CCA CGG GTG CAA GAG GAG CTG TGC CTG GTT 871 Gly Pro Ala Pro Arg Val Gln Glu Glu Leu Cys Leu Val 255 260 265
35	TGC GGC GAC AGG GCC TCC GGC TAC CAC TAC AAC GCC CTC 910 Cys Gly Asp Arg Ala Ser Gly Tyr His Tyr Asn Ala Leu 270 275
40	ACC TGT GAG GGC TGC AAG GGG TTC TTT CGA CGC AGC GTT 949 Thr Cys Glu Gly Cys Lys Gly Phe Phe Arg Arg Ser Val 280 285 290
45	ACG AAG AGC GCC GTC TAC TGC TGC AAG TTC GGG CGC GCC 988 Thr Lys Ser Ala Val Tyr Cys Cys Lys Phe Gly Arg Ala 295 300 305
50	TGC GAA ATG GAC ATG TAC ATG AGG CGA AAG TGT CAG GAG 1027 Cys Glu Met Asp Met Tyr Met Arg Arg Lys Cys Gln Glu 310 315
55	TGC CGC CTG AAA AAG TGC CTG GCC GTG GGT ATG CGG CCG 1066 Cys Arg Leu Lys Lys Cys Leu Ala Val Gly Met Arg Pro 320 325 330
60	GAA TGC GTC GTC CCG GAG AAC CAA TGT GCG ATG AAG CGG 1105 Glu Cys Val Val Pro Glu Asn Gln Cys Ala Met Lys Arg 335 340
65	CGC GAA AAG AAG GCC CAG AAG GAG AAG GAC AAA ATG ACC 1144 Arg Glu Lys Lys Ala Gln Lys Glu Lys Asp Lys Met Thr 345 350 355
70	ACT TCG CCG AGC TCT CAG CAT GGC GGC AAT GGC AGC TTG 1183 Thr Ser Pro Ser Ser Gln His Gly Gly Asn Gly Ser Leu 360 365 370
75	GCC TCT GGT GGC GGC CAA GAC TTT GTT AAG AAG GAG ATT 1222 Ala Ser Gly Gly Gly Gln Asp Phe Val Lys Lys Glu Ile 375 380

	CTT GAC CTT ATG ACA TGC GAG CCG CCC CAG CAT GCC ACT 1261
	Leu Asp Leu Met Thr Cys Glu Pro Pro Gln His Ala Thr
	385 390 395
5	ATT CCG CTA CTA CCT GAT GAA ATA TTG GCC AAG TGT CAA 1300
	Ile Pro Leu Leu Pro Asp Glu Ile Leu Ala Lys Cys Gln
	400 405
10	GCG CGC AAT ATA CCT TCC TTA ACG TAC AAT CAG TTG GCC 1339
	Ala Arg Asn Ile Pro Ser Leu Thr Tyr Asn Gln Leu Ala
	410 415 420
15	GTT ATA TAC AAG TTA ATT TGG TAC CAG GAT GGC TAT GAG 1378
	Val Ile Tyr Lys Leu Ile Trp Tyr Gln Asp Gly Tyr Glu
	425 430 435
20	CAG CCA TCT GAA GAG GAT CTC AGG CGT ATA ATG AGT CAA 1417
	Gln Pro Ser Glu Glu Asp Leu Arg Arg Ile Met Ser Gln
	440 445
25	CCC GAT GAG AAC GAG AGC CAA ACG GAC GTC AGC TTT CGG 1456
	Pro Asp Glu Asn Glu Ser Gln Thr Asp Val Ser Phe Arg
	450 455 460
30	CAT ATA ACC GAG ATA ACC ATA CTC ACG GTC CAG TTG ATT 1495
	His Ile Thr Glu Ile Thr Ile Leu Thr Val Gln Leu Ile
	465 470
35	GTT GAG TTT GCT AAA GGT CTA CCA GCG TTT ACA AAG ATA 1534
	Val Glu Phe Ala Lys Gly Leu Pro Ala Phe Thr Lys Ile
	475 480 485
40	CCC CAG GAG GAC CAG ATC ACG TTA CTA AAG GCC TGC TCG 1573
	Pro Gln Glu Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser
	490 495 500
45	TCG GAG GTG ATG ATG CTG CGT ATG GCA CGA CGC TAT GAC 1612
	Ser Glu Val Met Met Leu Arg Met Ala Arg Arg Tyr Asp
	505 510
50	CAC AGC TCG GAC TCA ATA TTC TTC GCG AAT AAT AGA TCA 1651
	His Ser Ser Asp Ser Ile Phe Phe Ala Asn Asn Arg Ser
	515 520 525
55	TAT ACG CGG GAT TCT TAC AAA ATG GCC GGA ATG GCT GAT 1690
	Tyr Thr Arg Asp Ser Tyr Lys Met Ala Gly Met Ala Asp
	530 535
60	AAC ATT GAA GAC CTG CTG CAT TTC TGC CGC CAA ATG TTC 1729
	Asn Ile Glu Asp Leu Leu His Phe Cys Arg Gln Met Phe
	540 545 550
65	TCG ATG AAG GTG GAC AAC GTC GAA TAC GCG CTT CTC ACT 1768
	Ser Met Lys Val Asp Asn Val Glu Tyr Ala Leu Leu Thr
	555 560 565
70	GCC ATT GTG ATC TTC TCG GAC CGG CCG GGC CTG GAG AAG 1807
	Ala Ile Val Ile Phe Ser Asp Arg Pro Gly Leu Glu Lys
	570 575
75	GCC CAA CTA GTC GAA GCG ATC CAG AGC TAC TAC ATC GAC 1846
	Ala Gln Leu Val Glu Ala Ile Gln Ser Tyr Tyr Ile Asp
	580 585 590

	ACG	CTA	CGC	ATT	TAT	ATA	CTC	AAC	CGC	CAC	TGC	GGC	GAC	1885
	Thr	Leu	Arg	Ile	Tyr	Ile	Leu	Asn	Arg	His	Cys	Gly	Asp	
				595					600					
5	TCA	ATG	AGC	CTC	GTC	TTC	TAC	GCA	AAG	CTG	CTC	TCG	ATC	1924
	Ser	Met	Ser	Leu	Val	Phe	Tyr	Ala	Lys	Leu	Leu	Ser	Ile	
	605					610					615			
10	CTC	ACC	GAG	CTG	CGT	ACG	CTG	GGC	AAC	CAG	AAC	GCC	GAG	1963
	Leu	Thr	Glu	Leu	Arg	Thr	Leu	Gly	Asn	Gln	Asn	Ala	Glu	
			620					625					630	
15	ATG	TGT	TTC	TCA	CTA	AAG	CTC	AAA	AAC	CGC	AAA	CTG	CCC	2002
	Met	Cys	Phe	Ser	Leu	Lys	Leu	Lys	Asn	Arg	Lys	Leu	Pro	
				635						640				
20	AAG	TTC	CTC	GAG	GAG	ATC	TGG	GAC	GTT	CAT	GCC	ATC	CCG	2041
	Lys	Phe	Leu	Glu	Glu	Ile	Trp	Asp	Val	His	Ala	Ile	Pro	
		645					650					655		
25	CCA	TCG	GTC	CAG	TCG	CAC	CTT	CAG	ATT	ACC	CAG	GAG	GAG	2080
	Pro	Ser	Val	Gln	Ser	His	Leu	Gln	Ile	Thr	Gln	Glu	Glu	
				660					665					
30	AAC	GAG	CGT	CTC	GAG	CGG	GCT	GAG	CGT	ATG	CGG	GCA	TCG	2119
	Asn	Glu	Arg	Leu	Glu	Arg	Ala	Glu	Arg	Met	Arg	Ala	Ser	
	670					675					680			
35	GTT	GGG	GGC	GCC	ATT	ACC	GCC	GGC	ATT	GAT	TGC	GAC	TCT	2158
	Val	Gly	Gly	Ala	Ile	Thr	Ala	Gly	Ile	Asp	Cys	Asp	Ser	
			685					690					695	
40	GCC	TCC	ACT	TCG	GCG	GCG	GCA	GCC	GCG	GCC	CAG	CAT	CAG	2197
	Ala	Ser	Thr	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Gln	His	Gln	
					700					705				
45	CCT	CAG	CCT	CAG	CCC	CAG	CCC	CAA	CCC	TCC	TCC	CTG	ACC	2236
	Pro	Gln	Pro	Gln	Pro	Gln	Pro	Gln	Pro	Ser	Ser	Leu	Thr	
		710					715					720		
50	CAG	AAC	GAT	TCC	CAG	CAC	CAG	ACA	CAG	CCG	CAG	CTA	CAA	2275
	Gln	Asn	Asp	Ser	Gln	His	Gln	Thr	Gln	Pro	Gln	Leu	Gln	
				725				730						
55	CCT	CAG	CTA	CCA	CCT	CAA	CTG	CAA	GGT	CAA	CTG	CAA	CCC	2314
	Pro	Gln	Leu	Pro	Pro	Gln	Leu	Gln	Gly	Gln	Leu	Gln	Pro	
						740					745			
60	CAG	CTC	CAA	CCA	CAG	CTT	CAG	ACG	CAA	CTC	CAG	CCA	CAG	2353
	Gln	Leu	Gln	Pro	Gln	Leu	Gln	Thr	Gln	Leu	Gln	Pro	Gln	
			750					755					760	
65	ATT	CAA	CCA	CAG	CCA	CAG	CTC	CTT	CCC	GTC	TCC	GCT	CCC	2392
	Ile	Gln	Pro	Gln	Pro	Gln	Leu	Leu	Pro	Val	Ser	Ala	Pro	
						765				770				
70	GTG	CCC	GCC	TCC	GTA	ACC	GCA	CCT	GGT	TCC	TTG	TCC	GCG	2431
	Val	Pro	Ala	Ser	Val	Thr	Ala	Pro	Gly	Ser	Leu	Ser	Ala	
		775					780					785		
75	GTC	AGT	ACG	AGC	AGC	GAA	TAC	ATG	GGC	GGA	AGT	GCG	GCC	2470
	Val	Ser	Thr	Ser	Ser	Glu	Tyr	Met	Gly	Gly	Ser	Ala	Ala	
						790				795				

ATA GGA CCC ATC ACG CCG GCA ACC ACC AGC AGT ATC ACG 2509
 Ile Gly Pro Ile Thr Pro Ala Thr Thr Ser Ser Ile Thr
 800 305 810

5 GCT GCC GTT ACC GCT AGC TCC ACC ACA TCA GCG GTA CCG 2548
 Ala Ala Val Thr Ala Ser Ser Thr Thr Ser Ala Val Pro
 815 820 825

10 ATG GGC AAC GGA GTT GGA GTC GGT GTT GGG GTG GGC GGC 2587
 Met Gly Asn Gly Val Gly Val Gly Val Gly Val Gly Gly
 830 835

15 AAC GTC AGC ATG TAT GCG AAG CCC CAG ACG GCG ATG GCC 2626
 Asn Val Ser Met Tyr Ala Lys Pro Gln Thr Ala Met Ala
 840 845 850

20 TTG ATG GGT GTA GCC CTG CAT TCG CAC CAA GAG CAG CTT 2665
 Leu Met Gly Val Ala Leu His Ser His Gln Glu Gln Leu
 855 860

ATC GGG GGA GTG GCG GTT AAG TCG GAG CAC TCG ACG ACT 2704
 Ile Gly Gly Val Ala Val Lys Ser Glu His Ser Thr Thr
 865 870 875

25 GCA TAG CAGGCGCAGA GTCAGCTCCA CCAACATCAC CACCACAACA 2750
 Ala
 878

30 TCGACGTCCT GCTGGAGTAG AAAGCGCAGC TGAACCCACA CAGACATAGG 2800

GGAAATGGGG AAGTTCTCTC CAGAGAGTTC GAGCCGAACT AAATAGTAAA 2850

35 AAGTGAATAA TTAATGGACA AGCGTAAAAT GCAGTTATTT AGTCTTAAGC 2900

CTGCAAATAT TACCTATTAT TCATACAAAT TAACATATAA TACAGCCTAT 2950

40 TAACAATTAC GCTAAAGCTT 2970

Claims:

1. A method of inducing gene expression in a mammalian cell comprising contacting an ecdysteroid with an ecdysteroid receptor polypeptide within a mammalian cell, wherein said mammalian cell further contains a DNA binding sequence for said ecdysteroid receptor when in combination with its ligand ecdysteroid, wherein formation of a receptor-ligand-DNA binding sequence complex induces gene expression.
2. The method of claim 1 wherein said ecdysteroid does not contain a 25 hydroxyl group.
3. The method of claim 2 wherein said ecdysteroid contains an electron-withdrawing group at position 5.
4. The method of claim 2 wherein said ecdysteroid contains an electron-withdrawing group at position 11.
5. The method of claim 3 wherein said electron-withdrawing group is selected from the following: hydroxyl, ketone, sulphydryl, nitrate, nitrite, fluorine, bromine, iodine and chlorine.
6. The method of claim 4 wherein said electron-withdrawing group is selected from the following: hydroxyl, ketone, sulphydryl, nitrate, nitrite, fluorine, bromine, iodine and chlorine.
7. The method of claim 2 wherein said ecdysteroid is selected from the following: muristerone A, 5-dehydroxy muristerone A, 11-dehydroxy muristerone A and ponasterone.
8. The method of claim 1 wherein said DNA binding sequence further contains a DNA sequence encoding a eukaryotic gene.
9. The method of claim 8 wherein said heterologous eukaryotic gene encodes a polypeptide or nucleic acid selected from the following: cytokine, enzyme, structural polypeptide, DNA and RNA.
10. The method of claim 8 wherein said cytokine is selected from the following: growth hormone, insulin-like growth factors, interleukins, human growth hormone, N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), parathyroid hormone, and leutinizing hormone (LH), hemopoietic growth factor, hepatic growth factor, fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor-alpha and -beta, mullerian-inhibiting substance, mouse gonadotropin-

associated peptide, glucagon, inhibin, activin, vascular endothelial growth factor, integrin, thrombopoietin, erythropoietin, nerve growth factors such as NGF- β , platelet-growth factor, hemopoietic growth factor, tissue factor protein, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, transforming growth factors (TGF) such as TGF-alpha and

5 TGF-beta, insulin-like growth factor-I and -II, latency associated peptide, erythropoietin, osteoinductive factors, osteoinductive factors, interferon-alpha, -beta, and -gamma, the colony stimulating factor (CSF) M-CSF, GM-CSF, and G-CSF, interleukins (ILs) IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 and other polypeptide factors.

10 11. The method of claim 8 wherein said enzyme is selected from the following: tissue plasminogen activase (TPA), urokinase, elastase, adenosine deaminase, bombesin, factor IX, factor VIII, thrombin, enkephalinase, β -lactamase, superoxide dismutase, reverse transcriptase, RNase, DNase, enzymes catalyzing glycolysis, Krebs' cycle, gluconeogenesis, urea cycle, oxidative phosphorylation, the synthesis or degradation of purines, pyrimidines,

15 nucleic acid polymers, cholesterol, protein, glycogen, fatty acids, carbohydrate, phospholipids, glycoproteins or lipids.

12 The method of claim 8 wherein said structural polypeptide is selected from the following: lung surfactant, albumin, antibodies, carrier proteins, transport proteins,

20 glycoprotein hormone receptors, CD-4, insulin-like growth factor binding proteins, calcitonin, factor VIII, an antibody, protein A or D, rheumatoid factors, viral antigens, HIV envelope proteins GP120 and GP140, immunoglobulins, serum proteins and any cytoskeletal polypeptide.

25 13. The method of claim 8 wherein said nucleic acid is DNA and RNA.

14. The method of claim 1 wherein said mammalian cell is within a mammal.

15. The method of claim 15 wherein said mammal is a human.

30

16. A method of producing a desired protein comprising:

a) contacting an ecdysteroid with an ecdysteroid receptor polypeptide within a mammalian cell, wherein said mammalian cell further contains a chimeric DNA sequence comprising 1) a first DNA binding sequence having binding specificity for said

35 ecdysteroid receptor when in combination with its ligand ecdysteroid and 2) a second DNA sequence encoding a desired protein heterologous to said DNA binding sequence and under the transcriptional control of the ecdysteroid receptor-ligand complex; and

b) incubating said cell and producing said desired protein.

17. The method of claim 16 wherein said mammalian cell is in a mammal.
18. The method of claim 16 wherein said desired protein is selected from the following: cytokines, enzymes and structural polypeptides.
- 5 19. The method of claim 16 wherein said ecdysteroid is selected from the following: muristerone A, 5-dehydroxy muristerone A, 11-dehydroxy muristerone A and ponasterone.
- 10 20. A method of regulating endogenous or heterologous genes in transgenic mammals comprising:
- 15 a) contacting an ecdysteroid with an ecdysteroid receptor polypeptide within a mammalian cell, wherein said mammalian cell further contains a chimeric DNA sequence comprising 1) a first DNA binding sequence having binding specificity for said ecdysteroid receptor when in combination with its ligand ecdysteroid, and 2) a second DNA sequence encoding a desired gene product heterologous to said DNA binding sequence and under the transcriptional control of the ecdysteroid receptor-ligand complex; and
- b) incubating said cell and producing said desired gene product.
- 20 21. The method of claim 20 wherein said ecdysteroid receptor is one having binding affinity for muristerone A.
22. The method of claim 20 wherein said ecdysteroid is one having no hydroxyl group at position 25.
- 25 23. The method of claim 22 wherein said ecdysteroid is selected from the following: muristerone A, 5-dehydroxy muristerone A, 11-dehydroxy muristerone A and ponasterone.
- 30 24. A method of regulating gene expression in a mammalian cell containing a DNA sequence encoding a polypeptide heterologous to said mammalian cell and under the transcriptional control of an ecdysteroid receptor comprising:
- 35 (a) transforming said mammalian cell to express a ecdysteroid receptor fusion polypeptide wherein said fusion polypeptide contains a heterologous transactivation domain; and
- (b) contacting said transformed mammalian cell with an ecdysteroid that activates said ecdysteroid receptor.

25. The method of claim 24 wherein said transactivation domain is selected from the following: herpes virus VP16 acidic activation domain, *lexA*, *c-fos*, GAL4 and *c-myc*.

26. The method of claim 24 wherein said ecdysteroid is selected from the following:
5 muristerone A, 5-dehydroxy muristerone A, 11-dehydroxy muristerone A and ponasterone.

27. The method of claim 24 wherein said ecdysteroid receptor is DHR23 α .

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AAGCTTCTTG TCCCCAGCCG ACGCTAAGTG AACGGAAAAC GGCCACAAAA 50

CGGCGACTAT CGGCTGCCAG AGG ATG AAG EGG GGC TGG TCG 91
 Met Lys Arg Arg Trp Ser
 1 5

AAC AAC GGC GGC TTC ATG CGC CTA CCG GAG GAG TCG TCC 130
 Asn Asn Gly Gly Phe Met Arg Leu Pro Glu Glu Ser Ser
 10 15

TCG GAG GTC ACG TCC TCC TCG AAC GGG CTC GTC CTG CCC 169
 Ser Glu Val Thr Ser Ser Ser Asn Gly Leu Val Leu Pro
 20 25 30

TCG GGG GTG AAC ATG TCG CCC TCG TCG CTG GAC TCG CAC 208
 Ser Gly Val Asn Met Ser Pro Ser Ser Leu Asp Ser His
 35 40 45

GAC TAT TGC GAT CAG GAC CTT TGG CTC TGC GGC AAC GAG 247
 Asp Tyr Cys Asp Gln Asp Leu Trp Leu Cys Gly Asn Glu
 50 55

TCC GGT TCG TTT GGC GGC TCC AAC GGC CAT GGC CTA AAT 286
 Ser Gly Ser Phe Gly Gly Ser Asn Gly His Gly Leu Asn
 60 65 70

CAG CAG CAG CAG AGC GTC ATC ACG CTG GCC ATG CAC GGG 325
 Gln Gln Gln Gln Ser Val Ile Thr Leu Ala Met His Gly
 75 80

TGC TCC AGC ACT CTG CCC GCG CAG ACA ACC ATC ATT CCG 364
 Cys Ser Ser Thr Leu Pro Ala Gln Thr Thr Ile Ile Pro
 85 90 95

ATC AAC GGC AAC GCG AAT GGG AAT GGA GGC TCC ACC AAT 403
 Ile Asn Gly Asn Ala Asn Gly Asn Gly Gly Ser Thr Asn
 100 105 110

GGC CAA TAT GTG CCG GGT GCC ACC AAT CTG GGA GCG TTG 442
 Gly Gln Tyr Val Pro Gly Ala Thr Asn Leu Gly Ala Leu
 115 120

GCC AAC GGG ATG CTC AAT GGG GGC CTC AAT GGA ATG CAG 481
 Ala Asn Gly Met Leu Asn Gly Gly Leu Asn Gly Met Gln
 125 130 135

CAA CAG ATT CAG AAT GGC CAC GGC CTC ATC AAC TCC ACA 520
 Gln Gln Ile Gln Asn Gly His Gly Leu Ile Asn Ser Thr
 140 145

FIG. 1A-1

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ACG	CCC	TCA	ACG	CCG	ACC	ACC	CCG	CTC	CAC	CTT	CAG	CAG	559
Thr	Pro	Ser	Thr	Pro	Thr	Thr	Pro	Leu	His	Leu	Gln	Gln	
150					155					160			
AAC	CTG	GGG	GGC	GCG	GGC	GGC	GGC	GGT	ATC	GGG	GGA	ATG	598
Asn	Leu	Gly	Gly	Ala	Gly	Gly	Gly	Gly	Ile	Gly	Gly	Met	
		165					170					175	
GGT	ATT	CTT	CAC	CAC	GCG	AAT	GGC	ACC	CCA	AAT	GGC	CTT	637
Gly	Ile	Leu	His	His	Ala	Asn	Gly	Thr	Pro	Asn	Gly	Leu	
				180					185				
ATC	GGA	GTT	GTG	GGA	GGC	GGC	GGC	GGA	GTA	GGT	CTT	GGA	676
Ile	Gly	Val	Val	Gly	Gly	Gly	Gly	Gly	Val	Gly	Leu	Gly	
	190					195					200		
GTA	GGC	GGA	GGC	GGA	GTG	GGA	GGC	CTG	GGA	ATG	CAG	CAC	715
Val	Gly	Gly	Gly	Gly	Val	Gly	Gly	Leu	Gly	Met	Gln	His	
			205					210					
ACA	CCC	CGA	AGC	GAT	TCG	GTG	AAT	TCT	ATA	TCT	TCA	GGT	754
Thr	Pro	Arg	Ser	Asp	Ser	Val	Asn	Ser	Ile	Ser	Ser	Gly	
215					220					225			
CGC	GAT	GAT	CTC	TCG	CCT	TCG	AGC	AGC	TTG	AAC	GGA	TAC	793
Arg	Asp	Asp	Leu	Ser	Pro	Ser	Ser	Ser	Leu	Asn	Gly	Tyr	
		230					235					240	
TCG	GCG	AAC	GAA	AGC	TGC	GAT	GCG	AAG	AAG	AGC	AAG	AAG	832
Ser	Ala	Asn	Glu	Ser	Cys	Asp	Ala	Lys	Lys	Ser	Lys	Lys	
				245					250				
GGA	CCT	GCG	CCA	CGG	GTG	CAA	GAG	GAG	CTG	TGC	CTG	GTT	871
Gly	Pro	Ala	Pro	Arg	Val	Gln	Glu	Glu	Leu	Cys	Leu	Val	
	255					260					265		
TGC	GGC	GAC	AGG	GCC	TCC	GGC	TAC	CAC	TAC	AAC	GCC	CTC	910
Cys	Gly	Asp	Arg	Ala	Ser	Gly	Tyr	His	Tyr	Asn	Ala	Leu	
			270					275					
ACC	TGT	GAG	GGC	TGC	AAG	GGG	TTC	TTT	CGA	CGC	AGC	GTT	949
Thr	Cys	Glu	Gly	Cys	Lys	Gly	Phe	Phe	Arg	Arg	Ser	Val	
280					285					290			
ACG	AAG	AGC	GCC	GTC	TAC	TGC	TGC	AAG	TTC	GGG	CGC	GCC	988
Thr	Lys	Ser	Ala	Val	Tyr	Cys	Cys	Lys	Phe	Gly	Arg	Ala	
		295					300					305	
TGC	GAA	ATG	GAC	ATG	TAC	ATG	AGG	CGA	AAG	TGT	CAG	GAG	1027
Cys	Glu	Met	Asp	Met	Tyr	Met	Arg	Arg	Lys	Cys	Gln	Glu	
				310					315				

FIG. 1A-2

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TGC	CGC	CTG	AAA	AAG	TGC	CTG	GCC	GTG	GGT	ATG	CGG	CCG	1066
Cys	Arg	Leu	Lys	Lys	Cys	Leu	Ala	Val	Gly	Met	Arg	Pro	
320				325				330					
GAA	TGC	GTC	GTC	CCG	GAG	AAC	CAA	TGT	GCG	ATG	AAG	CGG	1105
Glu	Cys	Val	Val	Pro	Glu	Asn	Gln	Cys	Ala	Met	Lys	Arg	
335				340									
CGC	GAA	AAG	AAG	GCC	CAG	AAG	GAG	AAG	GAC	AAA	ATG	ACC	1144
Arg	Glu	Lys	Lys	Ala	Gln	Lys	Glu	Lys	Asp	Lys	Met	Thr	
345				350				355					
ACT	TCG	CCG	AGC	TCT	CAG	CAT	GGC	GGC	AAT	GGC	AGC	TTG	1183
Thr	Ser	Pro	Ser	Ser	Gln	His	Gly	Gly	Asn	Gly	Ser	Leu	
360				365				370					
GCC	TCT	GGT	GGC	GGC	CAA	GAC	TTT	GTT	AAG	AAG	GAG	ATT	1222
Ala	Ser	Gly	Gly	Gly	Gln	Asp	Phe	Val	Lys	Lys	Glu	Ile	
375				380									
CTT	GAC	CTT	ATG	ACA	TGC	GAG	CCG	CCC	CAG	CAT	GCC	ACT	1261
Leu	Asp	Leu	Met	Thr	Cys	Glu	Pro	Pro	Gln	His	Ala	Thr	
385				390				395					
ATT	CCG	CTA	CTA	CCT	GAT	GAA	ATA	TTG	GCC	AAG	TGT	CAA	1300
Ile	Pro	Leu	Leu	Pro	Asp	Glu	Ile	Leu	Ala	Lys	Cys	Gln	
400				405									
GCG	CGC	AAT	ATA	CCT	TCC	TTA	ACG	TAC	AAT	CAG	TTG	GCC	1339
Ala	Arg	Asn	Ile	Pro	Ser	Leu	Thr	Tyr	Asn	Gln	Leu	Ala	
410				415				420					
GTT	ATA	TAC	AAG	TTA	ATT	TGG	TAC	CAG	GAT	GGC	TAT	GAG	1378
Val	Ile	Tyr	Lys	Leu	Ile	Trp	Tyr	Gln	Asp	Gly	Tyr	Glu	
425				430				435					
CAG	CCA	TCT	GAA	GAG	GAT	CTC	AGG	CGT	ATA	ATG	AGT	CAA	1417
Gln	Pro	Ser	Glu	Glu	Asp	Leu	Arg	Arg	Ile	Met	Ser	Gln	
440				445									
CCC	GAT	GAG	AAC	GAG	AGC	CAA	ACG	GAC	GTC	AGC	TTT	CGG	1456
Pro	Asp	Glu	Asn	Glu	Ser	Gln	Thr	Asp	Val	Ser	Phe	Arg	
450				455				460					
CAT	ATA	ACC	GAG	ATA	ACC	ATA	CTC	ACG	GTC	CAG	TTG	ATT	1495
His	Ile	Thr	Glu	Ile	Thr	Ile	Leu	Thr	Val	Gln	Leu	Ile	
465				470									
GTT	GAG	TTT	GCT	AAA	GGT	CTA	CCA	GCG	TTT	ACA	AAG	ATA	1534
Val	Glu	Phe	Ala	Lys	Gly	Leu	Pro	Ala	Phe	Thr	Lys	Ile	
475				480				485					

FIG. 1A-3

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CCC	CAG	GAG	GAC	CAG	ATC	ACG	TTA	CTA	AAG	GCC	TGC	TCG	1573
Pro	Gln	Glu	Asp	Gln	Ile	Thr	Leu	Leu	Lys	Ala	Cys	Ser	
		490					495					500	
TCG	GAG	GTG	ATG	ATG	CTG	CGT	ATG	GCA	CGA	CGC	TAT	GAC	1612
Ser	Glu	Val	Met	Met	Leu	Arg	Met	Ala	Arg	Arg	Tyr	Asp	
				505					510				
CAC	AGC	TCG	GAC	TCA	ATA	TTC	TTC	GCG	AAT	AAT	AGA	TCA	1651
His	Ser	Ser	Asp	Ser	Ile	Phe	Phe	Ala	Asn	Asn	Arg	Ser	
	515					520					525		
TAT	ACG	CGG	GAT	TCT	TAC	AAA	ATG	GCC	GGA	ATG	GCT	GAT	1690
Tyr	Thr	Arg	Asp	Ser	Tyr	Lys	Met	Ala	Gly	Met	Ala	Asp	
			530					535					
AAC	ATT	GAA	GAC	CTG	CTG	CAT	TTC	TGC	CGC	CAA	ATG	TTC	1729
Asn	Ile	Glu	Asp	Leu	Leu	His	Phe	Cys	Arg	Gln	Met	Phe	
540					545					550			
TCG	ATG	AAG	GTG	GAC	AAC	GTC	GAA	TAC	GCG	CTT	CTC	ACT	1768
Ser	Met	Lys	Val	Asp	Asn	Val	Glu	Tyr	Ala	Leu	Leu	Thr	
		555					560					565	
GCC	ATT	GTG	ATC	TTC	TCG	GAC	CGG	CCG	GGC	CTG	GAG	AAG	1807
Ala	Ile	Val	Ile	Phe	Ser	Asp	Arg	Pro	Gly	Leu	Glu	Lys	
				570					575				
GCC	CAA	CTA	GTC	GAA	GCG	ATC	CAG	AGC	TAC	TAC	ATC	GAC	1846
Ala	Gln	Leu	Val	Glu	Ala	Ile	Gln	Ser	Tyr	Tyr	Ile	Asp	
	580					585					590		
ACG	CTA	CGC	ATT	TAT	ATA	CTC	AAC	CGC	CAC	TGC	GGC	GAC	1885
Thr	Leu	Arg	Ile	Tyr	Ile	Leu	Asn	Arg	His	Cys	Gly	Asp	
			595					600					
TCA	ATG	AGC	CTC	GTC	TTC	TAC	GCA	AAG	CTG	CTC	TCG	ATC	1924
Ser	Met	Ser	Leu	Val	Phe	Tyr	Ala	Lys	Leu	Leu	Ser	Ile	
605					610					615			
CTC	ACC	GAG	CTG	CGT	ACG	CTG	GGC	AAC	CAG	AAC	GCC	GAG	1963
Leu	Thr	Glu	Leu	Arg	Thr	Leu	Gly	Asn	Gln	Asn	Ala	Glu	
		620					625					630	
ATG	TGT	TTC	TCA	CTA	AAG	CTC	AAA	AAC	CGC	AAA	CTG	CCC	2002
Met	Cys	Phe	Ser	Leu	Lys	Leu	Lys	Asn	Arg	Lys	Leu	Pro	
				635					640				
AAG	TTC	CTC	GAG	GAG	ATC	TGG	GAC	GTT	CAT	GCC	ATC	CCG	2041
Lys	Phe	Leu	Glu	Glu	Ile	Trp	Asp	Val	His	Ala	Ile	Pro	
	645					650					655		

FIG. 1A-4

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CCA	TCG	GTC	CAG	TCG	CAC	CTT	CAG	ATT	ACC	CAG	GAG	GAG	2080
Pro	Ser	Val	Gln	Ser	His	Leu	Gln	Ile	Thr	Gln	Glu	Glu	
			660										
AAC	GAG	CGT	CTC	GAG	CGG	GCT	GAG	CGT	ATG	CGG	GCA	TCG	2119
Asn	Glu	Arg	Leu	Glu	Arg	Ala	Glu	Arg	Met	Arg	Ala	Ser	
670					675					680			
GTT	GGG	GGC	GCC	ATT	ACC	GCC	GGC	ATT	GAT	TGC	GAC	TCT	2158
Val	Gly	Gly	Ala	Ile	Thr	Ala	Gly	Ile	Asp	Cys	Asp	Ser	
		685					690					695	
GCC	TCC	ACT	TCG	GCG	GCG	GCA	GCC	GCG	GCC	CAG	CAT	CAG	2197
Ala	Ser	Thr	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Gln	His	Gln	
				700					705				
CCT	CAG	CCT	CAG	CCC	CAG	CCC	CAA	CCC	TCC	TCC	CTG	ACC	2236
Pro	Gln	Pro	Gln	Pro	Gln	Pro	Gln	Pro	Ser	Ser	Leu	Thr	
	710					715					720		
CAG	AAC	GAT	TCC	CAG	CAC	CAG	ACA	CAG	CCG	CAG	CTA	CAA	2275
Gln	Asn	Asp	Ser	Gln	His	Gln	Thr	Gln	Pro	Gln	Leu	Gln	
			725					730					
CCT	CAG	CTA	CCA	CCT	CAA	CTG	CAA	GGT	CAA	CTG	CAA	CCC	2314
Pro	Gln	Leu	Pro	Pro	Gln	Leu	Gln	Gly	Gln	Leu	Gln	Pro	
735					740					745			
CAG	CTC	CAA	CCA	CAG	CTT	CAG	ACG	CAA	CTC	CAG	CCA	CAG	2353
Gln	Leu	Gln	Pro	Gln	Leu	Gln	Thr	Gln	Leu	Gln	Pro	Gln	
		750					755					760	
ATT	CAA	CCA	CAG	CCA	CAG	CTC	CTT	CCC	GTC	TCC	GCT	CCC	2392
Ile	Gln	Pro	Gln	Pro	Gln	Leu	Leu	Pro	Val	Ser	Ala	Pro	
				765					770				
GTG	CCC	GCC	TCC	GTA	ACC	GCA	CCT	GGT	TCC	TTG	TCC	GCG	2431
Val	Pro	Ala	Ser	Val	Thr	Ala	Pro	Gly	Ser	Leu	Ser	Ala	
	775					780					785		
GTC	AGT	ACG	AGC	AGC	GAA	TAC	ATG	GGC	GGA	AGT	GCG	GCC	2470
Val	Ser	Thr	Ser	Ser	Glu	Tyr	Met	Gly	Gly	Ser	Ala	Ala	
			790					795					
ATA	GGA	CCC	ATC	ACG	CCG	GCA	ACC	ACC	AGC	AGT	ATC	ACG	2509
Ile	Gly	Pro	Ile	Thr	Pro	Ala	Thr	Thr	Ser	Ser	Ile	Thr	
800					805					810			
GCT	GCC	GTT	ACC	GCT	AGC	TCC	ACC	ACA	TCA	GCG	GTA	CCG	2548
Ala	Ala	Val	Thr	Ala	Ser	Ser	Thr	Thr	Ser	Ala	Val	Pro	
		815					820					825	

FIG. 1A-5

SUBSTITUTE SHEET

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ATG GGC AAC GGA GTT GGA GTC GGT GTT GGG GTG GGC GGC 2587
 Met Gly Asn Gly Val Gly Val Gly Val Gly Val Gly Gly
 830 835

AAC GTC AGC ATG TAT GCG AAG CCC CAG ACG GCG ATG GCC 2626
 Asn Val Ser Met Tyr Ala Lys Pro Gln Thr Ala Met Ala
 840 845 850

TTG ATG GGT GTA GCC CTG CAT TCG CAC CAA GAG CAG CTT 2665
 Leu Met Gly Val Ala Leu His Ser His Gln Glu Gln Leu
 855 860

ATC GGG GGA GTG GCG GTT AAG TCG GAG CAC TCG ACG ACT 2704
 Ile Gly Gly Val Ala Val Lys Ser Glu His Ser Thr Thr
 865 870 875

GCA TAG CAGGCGCAGA GTCAGCTCCA CCAACATCAC CACCACAACA 2750
 Ala
 878

TCGACGTCCT GCTGGAGTAG AAAGCGCAGC TGAACCCACA CAGACATAGG 2800

GGAAATGGGG AAGTTCTCTC CAGAGAGTTC GAGCCGAACT AAATAGTAAA 2850

AAGTGAATAA TTAATGGACA AGCGTAAAAT GCAGTTATTT AGTCTTAAGC 2900

CTGCAAATAT TACCTATTAT TCATACAAAT TAACATATAA TACAGCCTAT 2950

TAACAATTAC GCTAAAGCTT 2970

FIG. 1A-6

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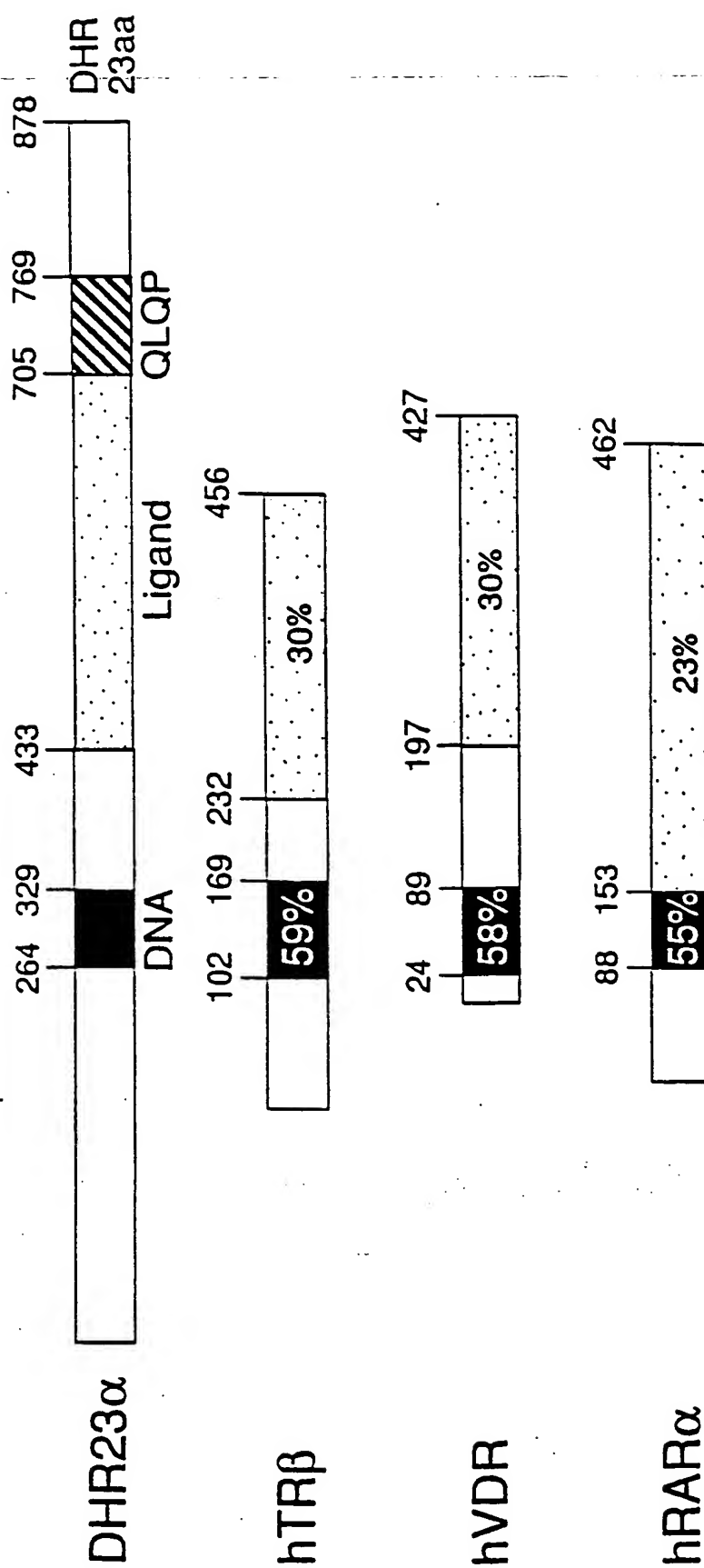


FIG. 1B

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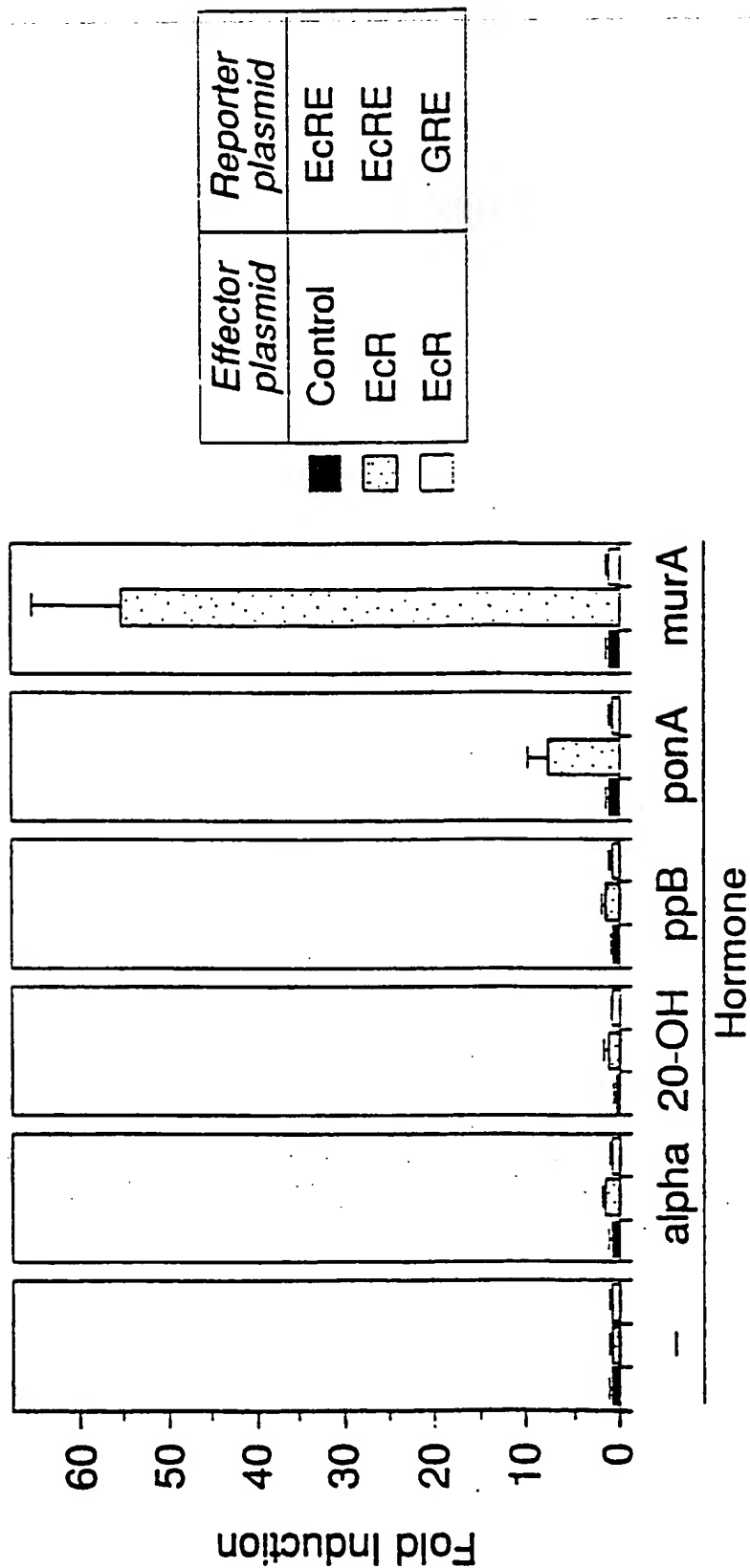
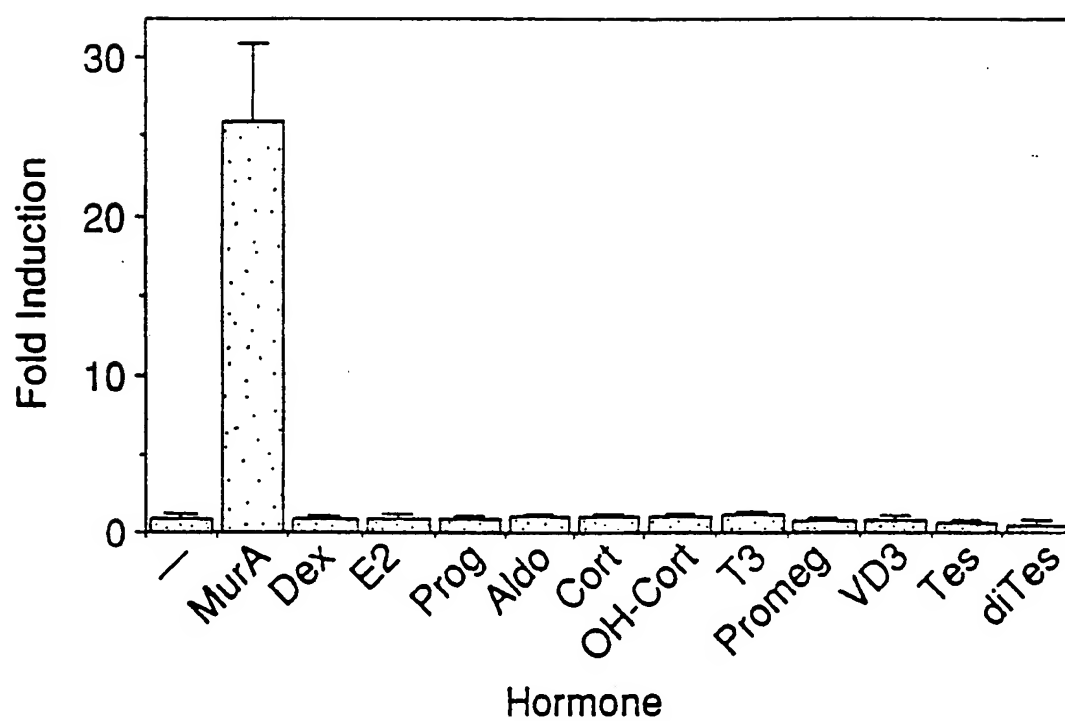


FIG. 2

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**FIG. 3**

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Lane	Receptor			Activation	DNA	Ligand
1	Ec	Ec	Ec		Ec Ec Ec	
2	G	G	G	G	G	G
3	G	G	Ec	G	G Ec	
4	V	G	Ec	V	G Ec	
5	G	E	G	G	E	G
6	G	E	Ec	G	E Ec	
7	G	X	G	G	X	G
8	G	X	Ec	G	X Ec	
9	V	X	Ec	V	X Ec	

FIG. 4A

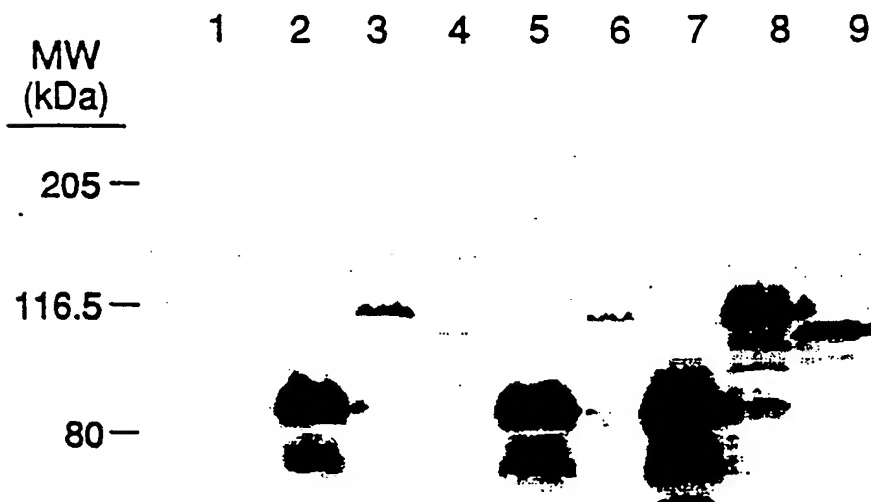


FIG. 4B

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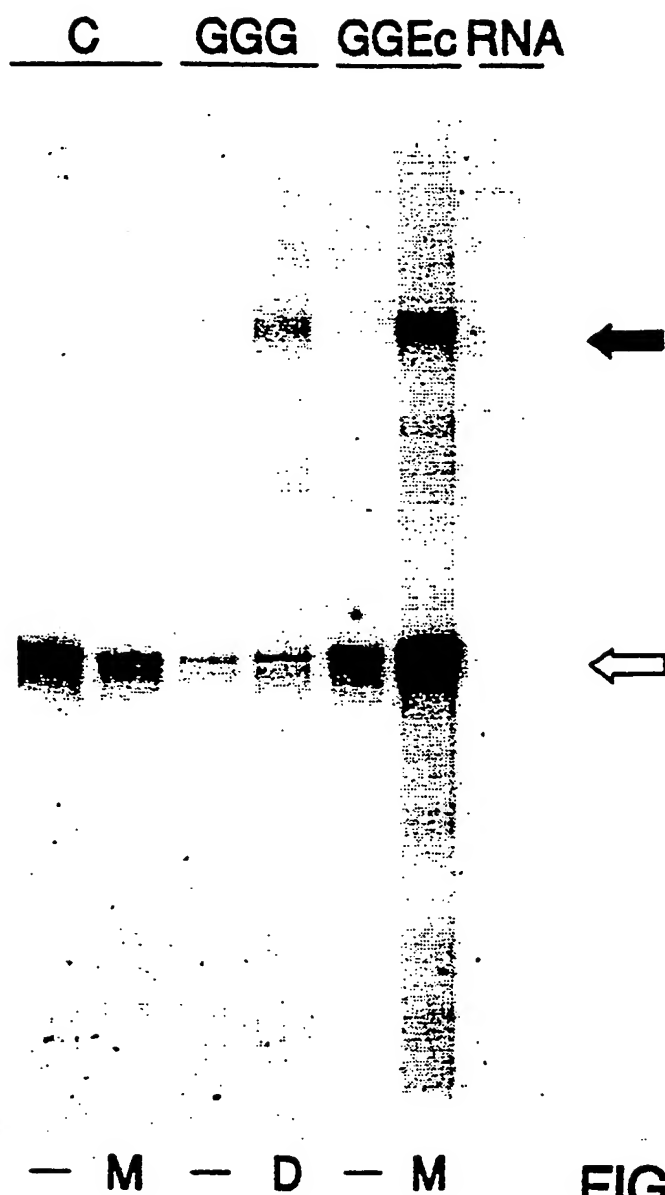


FIG. 5

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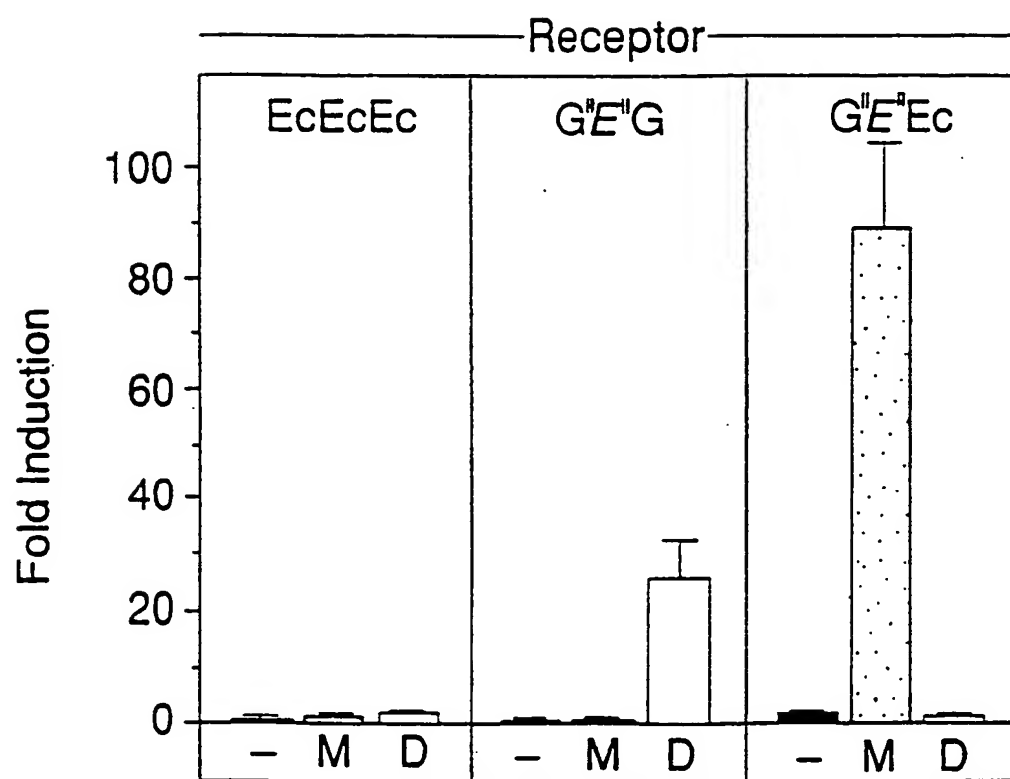


FIG. 6

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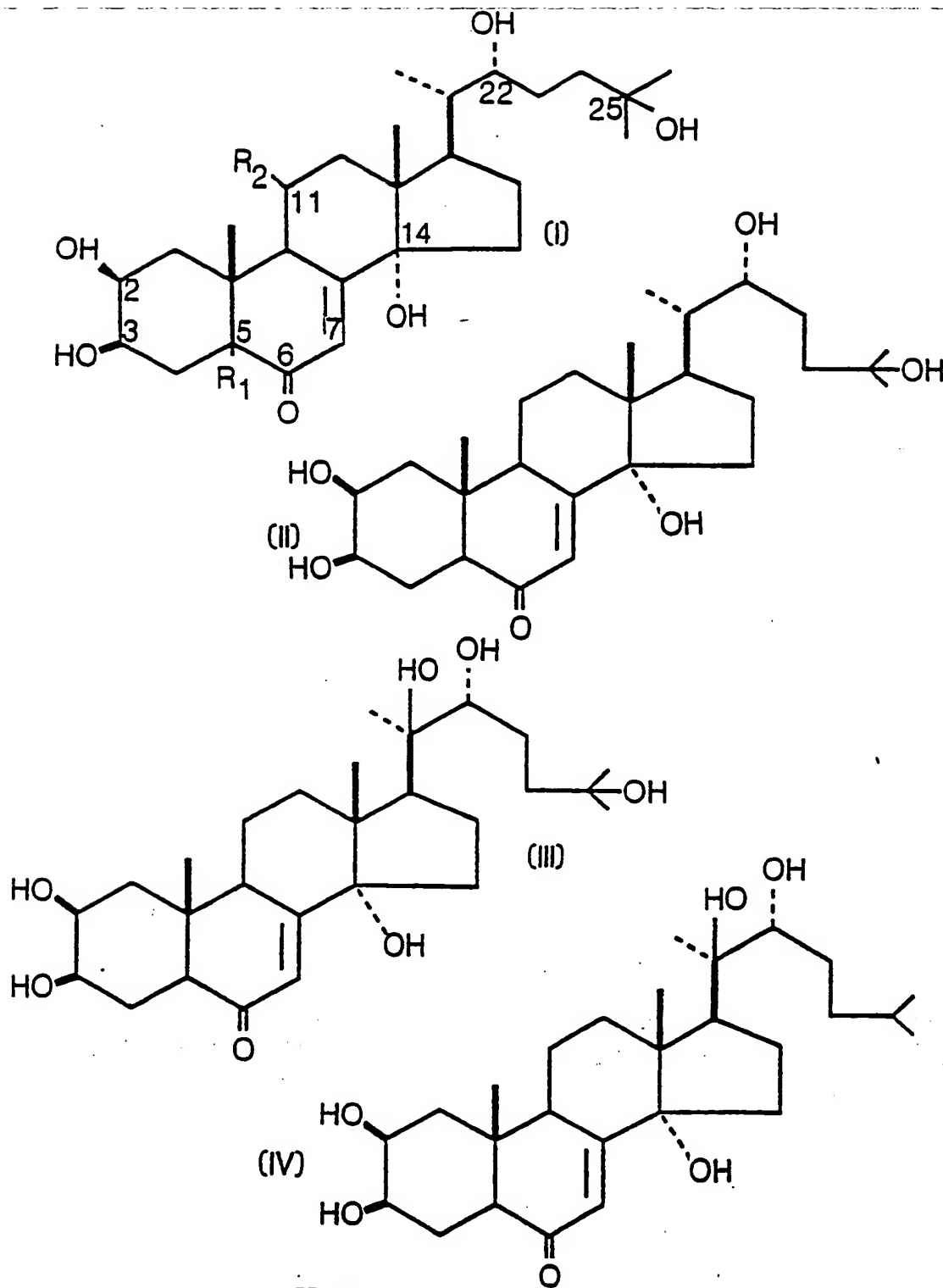


FIG. 7A

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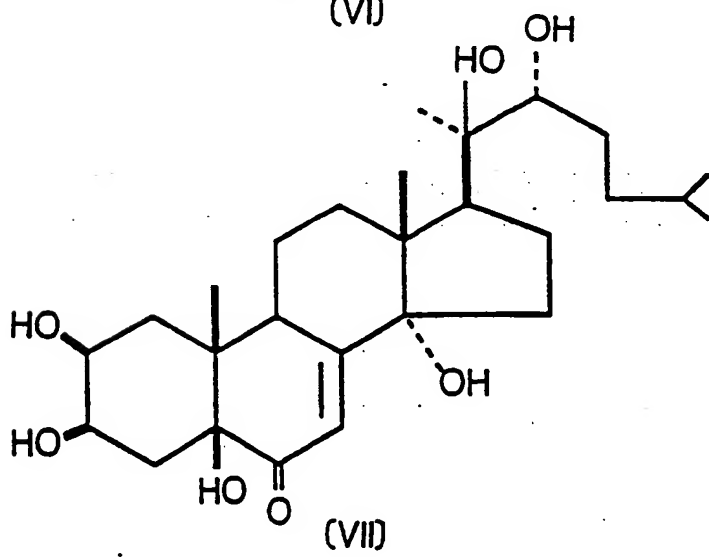
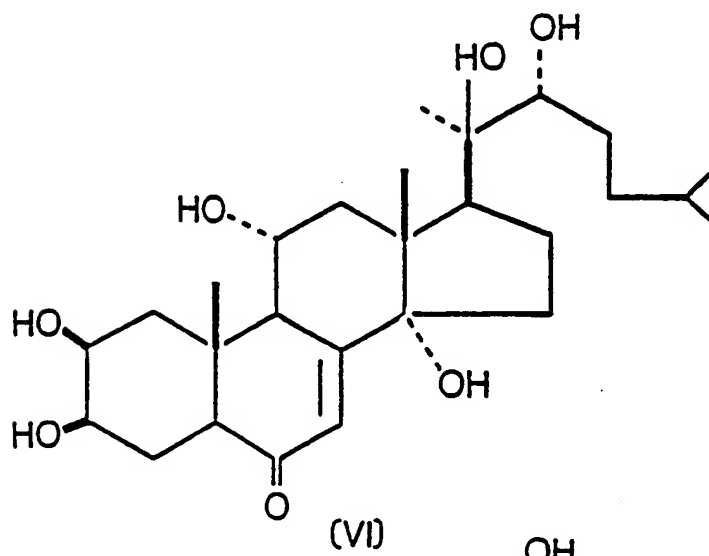
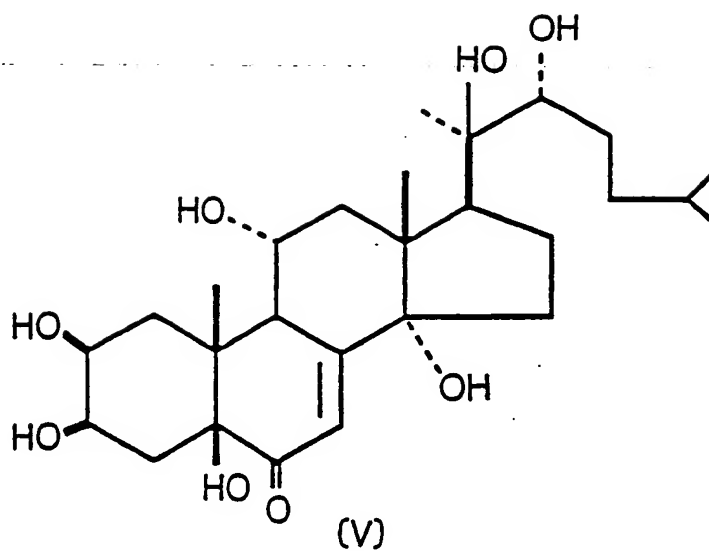


FIG. 7B

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/06391

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/85; C12N15/38;	C12N15/00; C12N15/63;	C12N15/62; C12N15/31 C12N15/12
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C07K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 89, July 1992, WASHINGTON US pages 6314 - 6318 CHRISTOPHERSON, K. S. ET AL. 'Ecdysteroid-dependent regulation of genes in mammalian cells by a Drosophila ecdysone receptor and chimeric transactivators' see the whole document ---	1-27
P,X	WO,A,9 113 167 (THE BOARD OF TRUSTEES OF LELAND STANFORD JR. UNIVERSITY) 5 September 1991 see the whole document ---	1,2,7-9, 16-20, 22-24,26
-/--		
<p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
24 NOVEMBER 1992		09.12.92
International Searching Authority		Signature of Authorized Officer
EUR PEAN PATENT OFFICE		CHAMBONNET F.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,Y	CELL vol. 67, 4 October 1991, CAMBRIDGE, MA US pages 59 - 77 KOELLE, M.R. ET AL. 'The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily' see the whole document ---	1,8-18, 20,24
Y	EP,A,0 244 221 (GENENTECH) 4 November 1987 see the whole document ---	1,8-18, 20,24
Y	WO,A,9 014 356 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 29 November 1990 see the whole document -----	1,8-18, 20,24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/06391

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: For claims 14 to 27 as far as they are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9206391
SA 63314

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 24/11/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9113167	05-09-91	AU-A- 7492291	18-09-91
EP-A-0244221	04-11-87	US-A- 4859609	22-08-89
		JP-A- 62272990	27-11-87
		US-A- 5030576	09-07-91
WO-A-9014356	29-11-90	AU-A- 5824890	18-12-90
		CA-A- 2057049	27-11-90
		EP-A- 0473716	11-03-92

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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